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(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 January 2003 (16.01.2003)

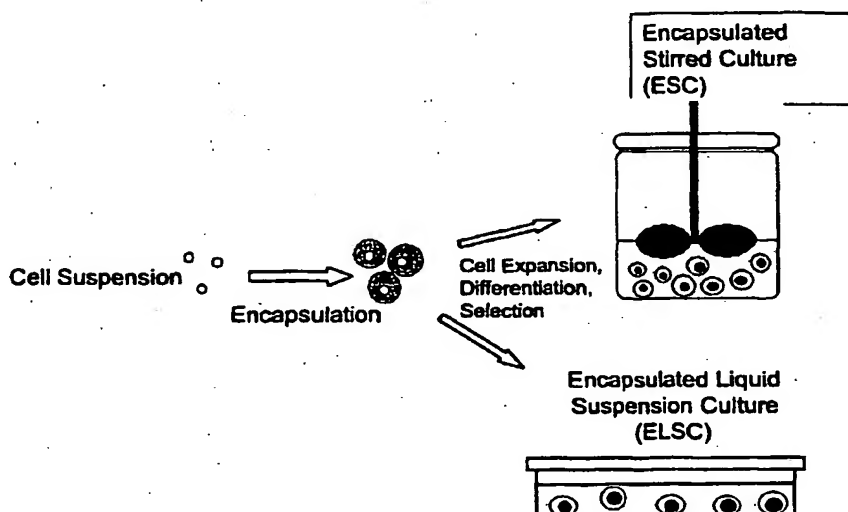
PCT

(10) International Publication Number
WO 03/004626 A1

- (51) International Patent Classification⁷: C12N 5/06, C12M 3/02, A01K 67/027
- (21) International Application Number: PCT/CA02/01022
- (22) International Filing Date: 4 July 2002 (04.07.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
2,351,156 4 July 2001 (04.07.2001) CA
60/302,706 5 July 2001 (05.07.2001) US
10/189,642 3 July 2002 (03.07.2002) US
- (71) Applicant (for all designated States except US): CARDION AG [DE/DE]; IPR, Max-Planck-Str. 15a, 40699 Erkrath (DE).
- (71) Applicants and
(72) Inventors: ZANDSTRA, Peter, W. [CA/CA]; 84 Alcina Avenue, Toronto, Ontario M6G 2E6 (CA). DANG, Stephen [CA/CA]; 58 Ambrose Road, North York, Ontario M2K 2W9 (CA).
- (74) Agent: BERESKIN & PARR; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report

[Continued on next page]

(54) Title: A BIOPROCESS FOR THE GENERATION OF CELLS DERIVED FROM SPHEROID-FORMING CELLS



(57) Abstract: The present inventors identified aggregation of embryonic stem cells and embryoid bodies (EBs) as the cause of the difficulty in generating large numbers of the embryonic stem cells (ES) cell-derived tissues. To counter this, the invention provides a novel bioprocess where aggregation of spheroid-forming cells, such as embryonic stem cells and spheroids, such as EBs is controlled, such as by encapsulation of within a matrix. As a result, EBs can be generated with high efficiency and cultured in high cell density, well-mixed systems. Well-mixed conditions facilitate measurement and control of the bulk media conditions and allow for the use of scalable bioreactor systems for clinical production of tissue. Therefore, the invention enables generation of ES cell-derived tissue on a clinical scale. The invention is also applicable to any spheroid-forming cells and other types of pluripotent cells.

WO 03/004626 A1



— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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TITLE: A BIOPROCESS FOR THE GENERATION OF CELLS FROM SPHEROID-FORMING CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application hereby claims the benefit of priority from Canadian Patent Application No. 2,351,156 filed July 4, 2001 and United States Provisional Patent Application No. 60/302,706 filed July 5, 2001, both entitled, "A Bioprocess For The Generation Of Pluripotent Cell Derived Cells And Tissues". Both of these references are herein incorporated by reference.

10

FIELD OF THE INVENTION

 The invention relates to a process for generating cells and tissues, preferably cells and tissues derived from pluripotent cells or spheroid-forming cells. In one embodiment the pluripotent cells are embryonic stem cells.

15 Products and applications resulting from said process are also encompassed within the scope of this application.

BACKGROUND OF THE INVENTION

 Tissue engineering, transplantation therapy, gene therapy, and drug discovery promise to revolutionize the medical paradigm and herald the coming of the "biotechnology era". However, development and implementation of these technologies depend upon cell-based approaches that require a plentiful supply of human cells: a supply that unfortunately does not currently exist. Therefore, identifying sources of human cells that can be

20 practically obtained in sufficient quantities is of paramount importance. Spheroid-forming cells, preferably pluripotent cells such as human embryonic stem cells (Thomson, Itskovitz-Eldor et al. 1998), neural stem cells (Clarke, Johansson, et al. 2000), multipotent adult progenitor cells (Schwartz, Reyes, et al. 2002), and others may be able to provide tissue in this manner.

30

 The potential utility of pluripotent cells, in particular, the utility of embryonic stem (ES) cells, is derived from their demonstrated ability to differentiate into any other cell type in the body (Evans and Kaufman, 1981). Derived from the early embryo, ES cells can be grown and manipulated in

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vitro. ES cells are pluripotent cells that have the ability to differentiate into any cell type in the body (Evans and Kaufman 1981). They also have the ability to grow and multiply indefinitely while maintaining their pluripotentiality. Theoretically, these traits make ES cells an unlimited source of any cell type and therefore a very attractive tissue source for other biotechnological applications (Zandstra and Nagy 2001).

Generation of various differentiated cell types (cardiac myocytes, hematopoietic cells, neurons, hepatocytes, and others) from murine ES cells (e.g., Wang, Clark et al. 1992; Rohwedel, Maltsev et al. 1994; Bain, Kitchens et al. 1995; Palacios, Golunski et al. 1995; Choi, Kennedy et al. 1998; Fleischmann, Bloch et al. 1998; Tropepe, Sibilio et al. 1999) as well as from human ES cells (e.g., Jones and Thomson 2000; Thomson et al. 1998; Mummery et al 2002; Levenberg et al. 2002; Reubinoff et al. 2001; Assady et al 2001) has been extensively reported.

In general, production of these various cell types from ES cells require a complex multi-step process, starting with the expansion of ES cells to adequate numbers. Next, ES cells would be differentiated to the desired cell type(s). Finally, the desired cells would be selected and purified from the remaining cells before they could be utilized for various applications.

In vitro, differentiating ES cells follow a reproducible temporal pattern of development that in many ways recapitulates early embryogenesis (Keller 1995). As ES cells and their derivatives proliferate and differentiate, they typically form spherical tissue-like structures (spheroids) called Embryoid Bodies (EBs). Other pluripotent stem cells such as neural stem cells have also been described to form spheroids. In the case of neural stem cells these spheroids are termed neurospheres (Kalyani, Hobson, and Rao, 1997). In the case of ES cells, over time, EBs increase in cell number and complexity as cells from the three embryonic germ layers are formed (Keller 1995). The ability to control and manipulate the formation of spheroids from pluripotent cells and other types of cells that are derived directly, or indirectly from human tissue, as well as the interactions between the spheroids and between the spheroid-forming cells (i.e., prevent or inhibit the aggregation of spheroids) would allow for the control of interactions between these cells during

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proliferation and differentiation, and thus be an important development for the design of technologies for the *in vitro* differentiation of pluripotent cells. For example, the generation of "chimeric spheroids" or "chimeric EBs" defined as multicellular, spherical structure comprising a mixture of differentiated cells and pluripotent cells (Perkins, 1998), has been shown to be an effective approach to influence the development of the cell types in from pluripotent cells (Clarke, Johansson et al. 2000).

Current ES cell differentiation systems include cultures initiated with an ES cell suspension in liquid media (LSC), or methylcellulose (semi-solid) media (MC), or attached to a surface in liquid media, or with multi-ES cell aggregates formed in "hanging drop" cultures (HD) where ES cells are aggregated in hanging drops for 2 days before transfer to liquid culture (see Figure 1). These systems are adequate for small-scale laboratory purposes but are not amenable to clinical production because of deficiency in three important areas: a) the ability to measure and control the extracellular environment, b) scalability, and c) cell density.

These systems are static and batch-style cultures that result in formation of spatial and temporal gradients in nutrients and metabolic products. These gradients make measurement and process control difficult because these processes depend on sampling measurements that need to reflect the conditions throughout the culture. The inability to control the cell culture environment will affect product purity and reproducibility, as well as the cell types that can be readily generated [e.g., for oxygen (Maltepe, Schmidt, et al. 1997), glucose (Soira, 2001) and many other biological and physicochemical factors (Zandstra and Nagy, 2001)]. In the case of methylcellulose culture, the semi-solid media also hinders measurement and manipulation. Regulatory approval for the use of any biotechnology product demands consistency in the method of production and in the final product itself (<http://www.fda.gov/cdrh/tisseng/te6.html>). This is not possible when measurement and control are impeded or absent.

Second, clinical or industrial scale production of cells from the current culture systems is not practical. The current systems are typically carried out in petri dishes where cells grow in only a thin layer of media. These systems

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are essentially two-dimensional with respect to the EBs; therefore, unrealistically large surface areas would be required for industrial scale production. It can be estimated that several billion cells would be need for applications such as cardiac cell therapy (Zandstra and Nagy, 2001); this
5 scale of cell production is highly impractical, if not impossible using current technologies.

Third, current liquid suspension cultures (LSC) require EBs to be cultured at relatively low density. Higher cell densities can be achieved in methylcellulose cultures, where semi-solid media reduces the likelihood of cell
10 aggregation, however media supplementation of methylcellulose cultures is troublesome and increased cell density would demand more frequent supplementation.

Therefore, there is a need to develop methods for efficient growth and differentiation of spheroid-forming cells, preferably pluripotent cells, such as
15 ES cells.

SUMMARY OF THE INVENTION

Because of the limitations reviewed above, one ideal differentiation culture system for clinical scale production of EBs or other spheroids would be
20 the stirred tank reactor or other scalable, well-mixed and controlled bioreactor systems such as a fluidized bed reactor. While the standard methods are limited to two-dimensional growth because of static conditions, a controlled bioreactor could suspend the cells evenly throughout the volume (in three-dimensions). This configuration of system can be easily scaled in size to
25 accommodate the need for increased production of cells. In addition, stirring ensures homogenous media conditions throughout the reactor that would facilitate measurement and control of the extracellular environment.

Despite the importance of stirred or other well-mixed bioreactors, to date there have been no reports of successful use of these systems for ES
30 cell differentiation culture. ES cells added directly to stirred culture quickly aggregate into large cell clumps within 24 hours and cell growth and differentiation within these clumps are severely impaired (Wartenberg et al. (1998)). However, it was not known whether aggregation was the cause or

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result of a different problem. Differentiation of ES cells in stirred culture is therefore a non-trivial task; however the ability to do so would greatly facilitate clinical and industrial scale production of cells for therapeutic purposes. ES cells cultured in stirred bioreactors fail to generate EBs in an efficient manner.

5 This patent application is based upon a novel technology that overcomes one or more of the limitations of the prior art: a) the ability to measure and control the extracellular environment, b) scalability, and c) cell density and allows for the scalable generation of cells and/or tissue derived from spheroid-forming, preferably pluripotent, preferably ES cells.

10 The present inventors have determined that ES cells cultured in stirred bioreactors fail to generate EBs in an efficient manner because the cells aggregate forming large cell clumps that results in poor cell proliferation and incomplete cell differentiation and that controlling aggregation can overcome this problem. As such, in one aspect, the present invention provides a process
15 to control cellular and spheroid aggregation of spheroid-forming cells, such as ES cells or neuronal stem cells, to enable the use of a stirred tank reactor or other scalable, well-mixed and controlled bioreactor systems such as a fluidized bed reactor or other liquid suspension system. The invention can also have benefits in other systems, especially liquid systems such as LSC.

20 In one embodiment, the invention provides a method of culturing spheroid-forming cells, preferably pluripotent cells, preferably ES cells, under controlled cell aggregation conditions that enable spheroid formation. In another embodiment the method is done in a bioreactor system by controlling cellular aggregation. In one embodiment the cells are differentiating cells. In
25 yet another embodiment, the invention provides a method of generating or obtaining cells derived from spheroid-forming cells cultured under controlled aggregation conditions. Although a preferred embodiment of the invention is with ES cells, the invention is not limited to ES cell derived cells and tissues. It is equally applicable to any spheroid-forming cell type, preferably pluripotent
30 cell type that can differentiate as a cell cluster or aggregate, or form spheroid bodies, such as adult pluripotent cells (Schwartz, Reyes, et al. 2002; Clarke, Johansson et al. 2000) embryonic germ cells (EG cells) (reviewed in Thomson and Odorico, 2000), early primitive ectoderm-like cells (EPL) (Pelton

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and Sharma, 2002), and neuronal stem cells. In one embodiment, the invention can also apply to other cell types where cell aggregation or control of the cell microenvironment is indicated. As such the embodiments of the invention as outlined below in relation to embryonic stem cells also apply to
5 other pluripotent cells where controlling aggregation of the cells at various points of expansion and or differentiation can enhance the efficiency of said expansion, spheroid formation or differentiation and are intended to be encompassed within the scope of the present invention.

In one embodiment, the invention provides a method of generating
10 cells derived from spheroid-forming or pluripotent cells comprising culturing the cells, for example pluripotent cells, such as ES cells, EG cells, EPL cells, and adult pluripotent cells (Schwartz, Reyes, et al. 2002), while controlling cellular and spheroid aggregation. In a preferred embodiment, the method is conducted under conditions that permit cell differentiation and proliferation. In
15 another embodiment, the cells are encapsulated to prevent aggregation with neighboring cells, spheroids or cells contained in separate capsules. In another embodiment the aggregation between specific cell types is controlled (to enable aggregation necessary for spheroid formation, where applicable) and the aggregation between spheroids of these cells is prevented. In yet
20 another embodiment the kinetics of aggregation is controlled.

In one embodiment, the invention provides a method of generating cells from spheroid-forming cells, preferably embryonic stem cells, comprising culturing the spheroid-forming, preferably embryonic stem cells under conditions that enable spheroid or embryoid body formation and cell
25 differentiation while controlling cell aggregation.

In a preferred embodiment, the spheroid-forming cells, such as embryonic stem cells are encapsulated to control cell aggregation such that each capsule will transiently contain and give rise to one spheroid or embryoid body. In another embodiment, each capsule contains a predetermined or
30 desired number of spheroid-forming or ES cells that are permitted to aggregate and together give rise to a single spheroid or EB. Preferably, the undifferentiated spheroid-forming cells, such as embryonic stem cells are first encapsulated to prevent aggregation between the cells contained within

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separate capsules. The encapsulated cells are then cultured under conditions that enable cell proliferation and optionally differentiation, leading to spheroid or embryoid body formation. In a more preferred embodiment, emulsification of cells with agar in inert silicon oil is used to encapsulate the cells. In a most preferred embodiment, this emulsification results in the generation of agarose microcapsules containing cells. In yet another embodiment the process of encapsulation allows for the control of spheroid-forming cell aggregation by interfering with cell surface receptor binding. In one embodiment, the process of encapsulation allows for the control of spheroid-forming or ES cell aggregation by interfering with E-cadherin mediated cell aggregation.

In another embodiment, the invention provides a method wherein the embryonic stem cells are cultured under conditions wherein embryoid bodies and/or differentiated embryonic stem cells can be formed. For example, in some ES cell lines multiple ES cells may be preferred for EB formation. Following these permissive steps, cells are transferred to conditions that prevent aggregation of embryoid bodies. In one embodiment, the method further comprises a step wherein the differentiated embryonic stem cells and/or tissues of interest are selected and harvested. In a preferred embodiment, the cells and/or tissues of interest are cardiomyocytes or cardiac tissue or hematopoietic cells or tissue or endothelial cells or tissues.

In another embodiment, the invention provides an embryonic stem cell derived cell culture obtained using the method of the invention. In a further embodiment, the invention provides embryonic stem cell derived cells and tissues obtained using the method of the invention.

In yet another embodiment the invention provides a method to identify factors, e.g. any variant, condition, substance, that affect embryonic stem cell differentiation and/or embryoid body formation, said method comprising culturing the embryonic stem cells while preventing embryoid body and embryonic stem cell aggregation, in the presence of the factor to be tested and then detecting the effect of the variant on embryonic stem cell differentiation and embryoid body formation. In a further embodiment, of said method, the effect on embryonic stem cell differentiation and embryoid body formation is compared to a control culture, preferably a negative control

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wherein embryonic stem cells are cultured under the same conditions except in the absence of the factor to be tested. The invention also provides a method of identifying factors that affect cell proliferation or differentiation in any cell culture by encapsulating said cells. The invention also encompassed
5 a method of preventing cell aggregation in any cell type by encapsulation.

Applications and uses of the method and initiating and resulting cells, tissues, cultures and bioreactors or culture systems are also encompassed within the scope of the present invention.

Other features and advantages of the present invention will become
10 apparent from the following detailed description. For instance where reference is made to embryoid bodies or embryonic stem cells, the invention can also be applied to spheroid-forming cells and spheroids. It should be understood, however, that the detailed description and the specific examples while
15 indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

20 The invention will now be described in relation to the non-limiting drawings in which:

Figure 1 is a schematic drawing of the current technologies used to culture pluripotent stem cells. (A) Hanging drop (HD) cultures typically consist of a
25 defined number of cells allowed to aggregate in small fluid volumes that hang from the tops of tissue culture dishes. Liquid suspension cultures (LSC) (B) are typically low-density suspension cultures of individual cells or aggregates of cells. When individual ES cells are put into the bottoms of 96 well plates to measure EB developments these cultures are termed single cell liquid
30 suspension cultures (scLSC) (C). Methycellulose (MC) (or similar solid or semi solid cultures) are cultures where cells are enveloped in semi- or semi-solid media that prevents aggregation, for example by preventing the collision of cells or cell aggregates due to its high viscosity (D).

Figure 2 is a schematic diagram of one embodiment of the method to control spheroid-forming cell and/or pluripotent cell and spheroid aggregation. Test cell populations (either individual cells or controlled aggregates of cells) are encapsulated, put inside a controlled stirred suspension bioreactor (Encapsulated Stirred Culture (ESC)), or in any type of encapsulated liquid suspension culture (ELSC) where, because of the encapsulation technology, they are prevented from aggregating, and allowed to proliferate and differentiate into the desired cell type.

Figure 3 is a graph illustrating the affect of initial ES cell density (i.e., number of ES cells per unit volume) on EB efficiency (ratio of number of cell aggregates to number of input ES cells) over time for A: liquid suspension culture (LSC) and B: methylcellulose (MC) culture. Cultures were initiated with ES cell densities: 10^2 , 10^3 , 10^4 , 10^5 cells/ml.

Figure 4 is a linear graph illustrating cells per EB (y-axis) over time (x-axis). EBs were initiated with: 1 (scLSC), 100 (100 cells per drop in the hanging drop cultures, HD100), and 1000 (1000 cells per drop in the hanging drop cultures, HD1000) ES cells/EB. Despite initial differences in cells/EB, all EBs grew to approximately the same size by day 12.

Figure 5 (A) illustrates the method of quantifying degree of aggregation (DOA) between two EBs, defined as the ratio of the diameter of the interface to the overall length of the two EB system. DOA is calculated by dividing the interface diameter by the overall length of the two EB system, yielding a scale between 0 (no aggregation) and 1 (fully aggregated). (B) A representative photomicrograph series of two EBs aggregating over time is shown with corresponding DOA measurements at each time interval. Two types of ES cell derived EBs were used in this experiment, one that was not fluorescent and one that was constitutively fluorescent (light EB in the photomicrograph)).

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Figure 6 is a linear graph illustrating the effects of the chemical additives cytochalasin D (CytoD), mitomycin C (MitoC), E-cadherin blocking peptide (ECadAB), and a control, on the aggregation kinetics, measure using the DOA, of EBs. A representative photo taken at 24 hours of culture is shown for each treatment group.

Figure 7 panel A is a bar graph illustrating percent E-cadherin expressing cells (y-axis) versus time (x-axis). Figure 7 panel B is a linear graph illustrating aggregation kinetics of EBs differentiated for 0, 2, 4, and 6 days.

Figure 8 panel A are photographs of ES cells encapsulated inside agarose microcapsules designed to have specific diameters. Shown are capsules of mean diameter 35 50, and 85 μm . The scale is indicated by the straight lines at the bottom of each photograph which each represent 100 μm . In some capsules single ES cells were encapsulated ((35 and 50 μm capsules), while in other instances, a plurality of ES cells were encapsulated (85 μm capsules). Figure 8 panel B is a photograph series of differentiating ES cells forming EBs inside the agarose microcapsules. Day 0 shows encapsulated ES cells, day 2 shows day 2 EBs, day 4 shows EBs emerging, as desired, from the agarose microcapsules.

Figure 9 is a series of flow cytometry plots showing phenotypic detection of endothelial cells generated from encapsulated ES cells grown in stirred suspension bioreactors using flow cytometric based cell surface expression of various endothelial cell markers. Figure 9 panel A shows cells expressing Flk-1 (y-axis) and CD34 (x-axis). Cells expressing either Flk-1 or CD34 were gated and the percent of cells expressing these markers is indicated. Figure 9 panel B shows CD31 expression by ungated cells. Figure 9 panel C shows CD31 expression by gated cells. CD34 and/or Flk-1 expression together with CD31 expression identifies endothelial cells.

Figure 10 Panel A shows kinetic changes of the percentage of the MF-20 positive cells during the course of differentiation and selection with G418 of

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transgene – containing ES cells cultured in stirred suspension bioreactors. Figure 10 panel B shows morphologic and structural analysis of bioreactor – grown ES cell-derived cardiomyocytes. The cells exhibit a mature sarcomeric organization and Z-banding (arrow). Figure 10 panel C shows a cross section of a “cardiac body” (a spheroid of ES cell derived cardiac cells) generated in bioreactors.

10 DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

“Adult stem cell” as used herein means an undifferentiated cell found in a tissue in an adult organism that can renew itself and can (with certain limitations) differentiate to yield specialized cell types of the tissue from which it originated or to yield specialized cell types in other tissues and organs.

“Pluripotent cell” as used herein refers to a cell that can self renew and differentiate as a cell cluster or aggregate into one or more types of cells and/or tissue. Examples of such cells include but are not limited to embryonic stem cells, embryonic germ cells, early primitive ectoderm-like cells and derivatives, multiple adult progenitor cells, adult or embryonic neural stem cells (NSC) (Toma and Akhavan, 2001; Hitoshi, Tropepe, Sibilia, et al. 1999) adult mesenchymal stem cells (MSC), mesenchymal adult pluripotent stem cells, sometimes referred to as MAPC (Schwartz, Reyes, et al. 2002), ductal stem cells (Suzuk, Zheng et al. 2002), muscle derived stem cells (McKinney-Freeman, Jackson et al. 2002).

“Embryonic stem cell” as used herein refers to pluripotent cells derived from the inner cell mass of a blastocyst, or derived by other methodologies (Jones and Thomson (2000)) and shown to be able to contribute to development of multiple tissues.

“Embryonic stem cell derived cells and tissues” as used herein refers to any cells or tissues that are derived from embryonic stem cells. The term “pluripotent cell derived cells and tissues” as used herein refers to any cells or tissues that are derived from pluripotent cells or tissues. The term “spheroid-

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forming cell derived cells and tissues" as used herein refers to any cells or tissues that are derived from spheroid-forming cells or tissues.

"Spheroid-forming cell" as used herein means cells that form multi-cellular aggregates consisting of more than a single cell when cultured in suspension

5 "Spheroid" as used herein means a cellular structure consisting of more than a single cell that has initially developed from a single or from multiple cells.

"Embryoid Body" as used herein means a "Spheroid" which is derived from embryonic stem cells.

10 "Clonal Spheroid" as used herein means a cellular structure consisting of more than a single cell but which has been developed from a single cell.

"Stem cell" as used herein means an "adult stem cell" or a "pluripotent cell".

15 "Chimeric spheroid" is a "spheroid" made up of at least two distinct cell types. For example, in one embodiment it has been shown that the mixing ES cells with adult pluripotent cells can broaden the differentiation capacity of the adult pluripotent cells, for instance a chimeric spheroid can comprise two different pluripotent cell types, one an adult pluripotent cell (e.g. neuronal pluripotent cell) and the other an ES cell. In one embodiment the ES cell can induce the other cell type to differentiate into other cell types such as cardiac
20 cells.

DESCRIPTION

The present invention is directed to a new method for generating spheroid-forming cell and/or pluripotent cell derived cells and tissues, such as
25 for example cardiomyocytes, hematopoietic cells, endothelial cells, insulin producing beta cells, neuronal cells, glial cells, kidney cells, hepatocytes, vascular progenitor cells or derivatives (such as hematopoietic stem cells or endothelial cells), to name a few.

30 The present inventors have shown that as cell density increases, cell aggregation occurs more readily, resulting in lower cell expansion and impaired cell differentiation (also see Dang, et al. 2002). The present inventors have also hereby shown that cellular and spheroid aggregation is indeed the cause of the inability to culture spheroid-forming cells in a high

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density culture, such as in a liquid suspension culture (e.g. a stirred liquid bioreactor system). As such the inventors have shown that controlling cellular and/or spheroid aggregation can increase spheroid-forming efficiency.

Thus in one embodiment, the invention provides a method of
5 generating cells and/or tissues derived from spheroid-forming cells, preferably pluripotent cells comprising culturing spheroid-forming cells under controlled cell aggregation conditions. In another embodiment, such conditions enable spheroid formation in liquid suspension.

In a preferred embodiment, the invention provides a method for
10 efficient formation of spheroids or EBs and the culture of spheroid or EBs in suspension at higher cell densities. This is done by controlling cell aggregation. Thus, in another embodiment of the method of the invention, the spheroid-forming cells can be cultured under high density conditions. Although the invention will work at low cell density (even seeding the initial
15 culture with 1 cell), it can also work with an initial cell density of about 10^2 to about 10^6 cells/ml, preferably about 10^3 to about 10^6 cells/ml, more preferably from about 10^4 to about 10^6 cells/ml. The invention is not intended to be limited by such cell density values and are provided as a suitable example. These cells can then be cultured under controlled aggregation conditions to
20 yield to form spheroids. As one can start with a culture with more cells than the prior art, the resulting number of spheroids formed will be greater than in cultures commenced at a lower cell density. In one embodiment, each spheroid may be cultured to about 30,000 to 35,000 cells per spheroid, preferably about 30,000 to 46,000 cells per spheroid. It should be noted that
25 this is but one embodiment, and the invention is not limited to such numbers. A person skilled in the art could control conditions (such as time, temperature, media conditions, other environmental factors) to get lower or potentially higher numbers. Thus the present method permits generation of cells at an industrial scale.

30 In one embodiment, the invention provides a scalable method for generating spheroid-forming cell or pluripotent cell derived cells and tissues, such as spheroid or ES derived cells and tissues for various uses, such as for transplantation.

In another embodiment, the invention provides a method of culturing spheroid-forming cells, such as pluripotent cells in a bioreactor system where the culture conditions can be measured and controlled. In yet another embodiment, the invention provides a scalable and controllable culture of spheroid-forming cells, such as pluripotent cells by allowing them to be cultured in liquid suspension cultures, such as stirred bioreactors, such as stirred liquid bioreactors. This is done by controlling cell aggregation. This improves the efficiency of generation of differentiated cells.

In one embodiment, the bioreactor or culture system used in the invention is one that keeps the cells and/or spheroids in liquid suspension. In another embodiment, this culture system keeps the cells and/or spheroids in liquid suspension by stirring, but other methods or means can be used, such as by agitation of the system, use of various media or other environmental conditions.

The term "controlling cell aggregation" refers to the process of effectively modulating the extent and kinetics of cell aggregation such that an endpoint, not effectively attainable without such control, is achieved. As used herein in relation to cell and/or spheroid or EB aggregation, "controlling cell aggregation" means that cell aggregation can be permitted or prevented as desired. For example, in one aspect of the invention, aggregation of spheroid-forming cells, such as ES cells, sufficient to induce spheroid or EB formation is permitted, and aggregation between cells within a spheroid or EB is permitted. However aggregation between spheroid-forming cells or ES cells beyond that determined to induce spheroid or EB formation is prevented. Aggregation between separate EBs is also prevented.

Further, benefits of the invention (i.e. increasing efficiency of generating cells from spheroid-forming, pluripotent or ES cells) can be obtained with any degree of prevention of cellular and/or spheroid aggregation. Controlling aggregation may, in some application consist of preventing aggregation where the term "preventing" includes any inhibition of (i.e. that may prevent some, but not necessarily all) of spheroid-forming cell or pluripotent cell and/or spheroid or EB aggregation in a particular culture system. It would be appreciated by those skilled in the art upon reading the

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description herein that any prevention of spheroid, EB and/or spheroid-forming cell or pluripotent cell aggregation would improve cell yields in the culture system and/or cellular differentiation.

In one embodiment, "preventing, spheroid and/or ES cell and/or spheroid or EB aggregation" means that after spheroid-forming cells or ES cells are cultured to form spheroids or embryoid bodies, there may be cellular aggregation between cells within a spheroid or embryoid body but not between cells of different spheroids or embryoid bodies or between spheroid or embryoid body themselves.

In another aspect, the invention provides a method of generating cells derived from spheroid-forming cells wherein the spheroid-forming cells are cultured under conditions that enable spheroid formation, said conditions comprising culturing said spheroid-forming cells in liquid suspension under non-aggregating conditions.

Thus in one embodiment, the invention provides a method of culturing spheroid-forming cells or ES cells comprising culturing the cells under conditions that promote spheroid or EB formation, including controlled aggregation of spheroid-forming or ES cells, then preventing cell and spheroid or EB aggregation.

In another embodiment, the invention provides a method of controlling the production of spheroids from spheroid-forming cells by controlling the aggregation of such cells. In one embodiment, the spheroid-forming cells are pluripotent cells such as neural stem cells that form neurospheres, or are embryonic stem cell which form EBs.

In a preferred embodiment the method of the invention does not adversely affect spheroid or EB formation, cell growth, or cell differentiation. Thus in another preferred embodiment, the method does not adversely effect the delivery of desired nutrients, oxygen, or cytokines. In yet another embodiment, the method can further comprise culturing the spehroid-forming or ES cells under conditions that promote differentiation of the cells and spheroid or EB formation. For instance, ES cells can be cultured in a suitable base media (such as ES cell media) without LIF. In another embodiment, the cells can be cultured under conditions that do not promote cell differentiation

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or further differentiation. For instance ES cells can be cultured in a suitable base media (Such as ES cell media) with LIF. Thus the method of the invention, by adjusting culture conditions can generate differentiated cells or be used to expand the existing cell culture initially or at any point of the method. For instance, in one embodiment culture conditions can change be changed during the method to control differentiation conditions. Examples of culture conditions are described further below.

10

Examples of conditions that can be used in the present invention for culturing spheroid-forming cells, such as ES cells to control spheroid or EB and/or cell aggregation include but are not limited to:

15

(a) Methods for preventing physical association between cell aggregation molecules (i.e. encapsulation, such as described herein or Sefton et al. 1997).

(b) Methods that use reduced inorganic salt concentration as described in Boraston et al. 1992 and Ko et al. 2001.

20

(c) Methods that block surface aggregation molecules with specific peptides or other molecules, such as described in Burdsal et al. 1993

(d) Using genetically modified spheroid-forming or ES cells such as E-cadherin-null ES cells such as described in Larue et al. 1996 or methods that inhibit E-cadherin expression or antagonize or inhibit E-cadherin activity.

25

(e) Addition of agents that prevent cell aggregation such as dextran sulfate or other sulfated polyanions as described in Dee et al. 1997

30

(f) Methods that prevent cell aggregation by physically separating one or more spheroid-forming cells to enable spheroid formation in distinct compartments such as described in the single cell liquid suspension culture (scLSC) method in this patent without limitation to a single cell.

In one embodiment of the invention, the above methods can be used alone or in combination, ie, more than one method can be used to prevent

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spheroid, EB and/or spheroid-forming or ES cell and/or non-clonal spheroid-forming or non-clonal ES cell aggregation.

The selected spheroid-forming or ES cell culturing strategy preferably does not affect the development and differentiation of pluripotent cells,
5 preferably ES cells, or EBs in any undesired way.

In a preferred method of the invention, the spheroid-forming or ES cells are encapsulated. In one embodiment, the average number of initial cells encapsulated can be predetermined or conditions can be adjusted to get a desired number of cells per capsule (for instance the capsule matrix, cell
10 density, media or other environmental or capsule forming conditions). For instance, in one embodiment, the number of cells per capsule is 1-10, 5-10, 1-5, 5, or 1; but the invention is not limited by such examples. In one embodiment, the spheroid-forming or ES cell or cells can be encapsulated using methods such as described in Weaver et al. 1988 or Turcanu et al.
15 2001 and then cultured under conditions that promote spheroid or EB formation and cellular differentiation, such as described in Keller et al, 1995 or O'Shea et al. 1999. In another embodiment, the capsule comprises cells of at least two different cell types, such as an ES cell and an adult pluripotent cell to produce a chimeric spheroid.

20 A variety of methods can be used to encapsulate cells. Although, the Examples described herein use the Gel Microdrop Technique (GMD), patented and owned by One Cell Systems, Boston Massachusetts. The GMD technique was designed to encapsulate cells in agarose gel for the purpose of isolating individual cells with specific protein secretion profiles; however, the
25 present invention adapted the process for encapsulation of spheroid-forming or ES cells and controlling cell and spheroid or EB aggregation. It is important to note that the microencapsulation of spheroid-forming cells such as ES cells for the purposes of the present invention does not depend upon the GMD technique as any method of encapsulation could be used.

30 In one embodiment the size of the capsule can be controlled. In one aspect, this can determine the time at which a spheroid or EB emerges from the capsule. Preferably the size of the capsule is such that maintains the spheroid-forming cells and/or spheroids in the capsule until aggregation cell

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surface markers or other aggregating factors (molecules, proteins, etc..) are no longer expressed. For instance, under certain conditions, as noted in the present examples, for ES cells, EBs that emerge after about 4 days no longer express E-cadherin and thus do not have a tendency to aggregate. In one approach, varying the rate of emulsification during the encapsulation process can control the size of the microcapsule. The different capsule sizes produced under each of the conditions tested are shown in Table 3. Other factors that may control the size of the microcapsules include the addition of surfactants to the encapsulation solution, the ratio of encapsulation gel to liquid, and others factors that would be familiar to those practiced in the art upon reading this description. The efficiency of cell production could be affected by the timing of the emerging EB, as once the EB emerges from the capsule, that particular method of prevention of EB aggregation would be gone. The effect of any subsequent EB aggregation would depend on the stage of development of the emerged EB and any limitations in size (numbers of cells) of an EB. The size of the capsule could also effect efficiency of delivery of various growth or differentiation factors or any other factors or nutrients to the cells. For instance larger capsules would have greater surface in which such factors could pass through, or provide a substrate onto which one could functionalize bioactive molecules (Irvine et al, 1998). This could also be accomplished by controlling the porosity of the encapsulation matrix. In one embodiment, the method of the invention enables the culture of differentiating ES cells in conventional commercially available bioreactor systems that include the use of standard bioprocess equipment (probes, filters, etc).

The matrix of the capsule can be composed of a variety of substances. In one embodiment it is an agarose capsule. In another embodiment, the matrix should be biocompatible in that it should not have a detrimental effect on cell proliferation and/differentiation as desired. In yet another embodiment, the matrix should such that permits the passage or exposure of desired media components to the spheroid-forming cells or spheroid. This can be done by keeping certain components in the capsule and others out or by permitting the

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passage of certain components through the capsule matrix or by including certain components in the capsule matrix.

The encapsulation material chosen should prevent aggregation of cells contained in different capsules. Suitable material would include but is not limited to agarose, alginate, polymers such as poly HEMMA (Valbacka et al (2001) and others or matrices described in Weaver et al. (1988) and Sefton et al (1977).

The material selected for encapsulation should permit adequate delivery of any desired nutrients, cell culture media, growth factors, cytokines, or any other factors to promote desired spheroid-forming or ES cell growth, proliferation, differentiation or spheroid or EB formation, e.g., through functionalization of the encapsulation matrix, or by diffusion through the pores. The culture media can also include other factors such as low molecular weight molecules or drugs, such as retinoic acid or DMSO, as desired. Factors can also be included in the media for testing their effect on the cells. The material selected may depend on the factors that are to be delivered. Example of matrices that could be used are agarose, alginate, and polymers that support cell growth. Examples of suitable matrices are described in Weaver et al. (1988) and Sefton et al (1977). However, a person skilled in the art would be familiar with other suitable matrices upon reading this description.

Examples of culturing conditions that promote spheroid-forming cell or ES cell differentiation and spheroid or EB formation include but are not limited to conditions described in Keller et al. 1995 or O'Shea et al. 1999 or those outlined in Tables 10 or 11 and references noted therein to enhance generation of particular cell types. The spheroid-forming cell and other cells used in the invention can be genetically modified cells. The genetic modification can be for a variety of purposes including, to enhance cell selection by conferring particular identifying characteristic to the desired cell, to generate cells for gene or cell therapy, transplantation (for instance to reduce or prevent graft versus host disease and/or rejection) diagnostic and/or screening purposes. Other applications would be apparent to those skilled in the art.

- 20 -

More particularly, the conditions under which the ES cells are cultured for differentiation will depend on the type of ES cell derived cells and tissues desired. For instance, to obtain hematopoietic stem cells one would preferably culture the ES cells under certain conditions, such as described in Wiles and Johansson 1999; Rathjen et al. 1998; or Keller et al, 1993. On the other hand, if one wishes to obtain cardiomyocytes, the cells would be cultured under specific conditions, such as in the presence of specific cytokines, i.e. TGF- β (Dickson et al. 1993), or using a selectable genetic marker such as described for cardiac myocytes in Klug et al (1996). In vitro, ES cells have been shown to differentiate into a variety of cell types and tissues, including beta cells, cardiomyocytes, hepatic cells, kidney cells, neuronal cells, and hematopoietic cells. Various cell types and culturing conditions are described in Zandstra and Nagy, 2001 for general approaches; Wang et al, 1992 for endothelial cells; Rohwedel et al., 1994, Fleishman et al, 1998 and Mummery et al. 2002 for cardiac and muscle cells; Bain et al, 1995, Tropepe et al, 1999 and Reubinoff et al. 2001 for neuronal cells; Palacios et al, 1995 and Kyba et al., 2002 for hematopoietic stem cells; Choi et al; 1998 and Levenberg et al. 2002 for hematopoietic and endothelial cells. Assady et al. 2001 for insulin – producing cells. For other examples of culture conditions see Tables 10 and 11. Since the differentiation of the ES cells results in a complex mixture of all possible cell types an efficient enrichment protocol would be necessary to obtain a homogeneous population of specific cell types or tissues to be utilized for various purposes including the generation of surrogate cells for human therapies or for drug screen testing. This enrichment could be done by the introduction of a specific construct into ES cells, comprising a cell or tissue type specific promoter controlling the expression of a marker gene. The resulting transgenic ES cell(s) can be expanded, differentiated and selected to generate the desired cell type of interest using techniques known in the art, for instance as described in Klug et al (1996) for cardiac cells (WO 95/14079, 26 May 1995; U.S. Patent Nos. 5,733,727 and 6,015,671) or Soria et al (2000) or Soria et al (2001). The method is widely applicable to any cell type for which the tissue specific promoter is known. Table 9 provides some

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examples of promoters useful for the selection of specific cell lineages without limiting the number of useful promoters to that table.

Particular cell types can be selected for and/or harvested using a variety of methods, including cell surface receptors or other labeling, tagging
5 or monitoring or selection methods known in the art, such as expression of particular types of genes, proteins or levels of certain molecules.

In a further embodiment, the invention provides a method for the selection of specific cell types from spheroid-forming cells comprising the steps of:

- 10 i. introducing a reporter gene expressing vector into at least one spheroid-forming cell whereby a cell-type specific promoter is combined with an reporter gene, such that the reporter gene is expressed under the control of the cell-type specific promoter;
- 15 ii. culturing the spheroid-forming cell(s) , for example to expand the cells
- iii. differentiating spheroid-forming cell(s), for instance in accordance with the method of the present invention under controlled aggregation conditions.
- 20 iv. isolating and harvesting the specific cell-type based on the reporter gene expression

In one embodiment, the reporter gene is an antibiotic resistance gene and the cell-type of interest is isolated by the addition of an appropriate antibiotic in step (iii) or (iv).

25 In yet another embodiment, the method for the selection according to the reporter gene is selected from the group consisting of the Hygro mycin resistance gene (hph), the Zeocin resistance gene (Sh ble), the Puromycin resistance gene (pacA), and the Gentamycin of G418 resistance gene (aph).

In yet another embodiment, in the method for the selection the reporter
30 gene is selected from the group consisting of luciferase, green fluorescence protein, red fluorescence protein, and yellow fluorescence protein and the cells of interest are selected by fluorencent activated cell sorting (FACS).

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In yet another embodiment, in the method for the selection the reporter gene is selected from the group consisting of luciferase, green fluorescence protein, red fluorescence protein, yellow fluorescence protein, or a his-, myc-, or flag-tag ligated to a heterologous gene, or any heterologous gene which
5 when expressed is inserted into the cell surface and the cells of interest is isolated from the cultured cells by affinity purification.

In yet another embodiment, in the method for the selection, the cell type specific promoter is selected from those listed in Table 9 for cell types listed therein.

10 The initial undifferentiated spheroid-forming or ES cells used in the method can be obtained by methods known in the art. For instance one can use ES cells obtained directly from the inner cell mass of the embryo, or one can use ES cells that were cultured, i.e. expanded under conditions that preferably did not promote differentiation, such as described in Klug et al.
15 1996.

In another embodiment the method of the invention enables culture of differentiating spheroid-forming or ES cells in stirred 3-dimensional reactors with scalable volume.

20

In another aspect the invention provides a culture bioreactor for industrial production of cells derived from spheroid-forming cells comprising:

- 25 a. culturing spheroid-forming cells in accordance with the method of the invention under controlled aggregation, wherein the cells are cultured in a spheroid-forming cell suitable media under conditions that promote spheroid formation; while
b. inhibiting or preventing spheroid aggregation.

Such suitable media conditions are any conditions that promote spheroid formation, examples of which are provided herein.

30

In another embodiment, the invention provides a bioreactor for generating cells from spheroid-forming cells comprising a means for controlling conditions suitable for spheroid formation, such conditions comprising a means for controlling cellular aggregation and means for

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maintaining said cells and generated cells in suspension, such as means for preventing cellular aggregation.

5 In yet another embodiment the invention allows for measurement and control of the culture environment by enabling the use of stirred or other well-mixed bioreactors.

As a result of being able to better control the ES cell culture environment, the method of the invention enables one to better study various conditions to obtain particular differentiated ES cells and tissue (i.e. hematopoietic cells, cardiomyocytes, nerve cells, beta cells, hepatic cells, kidney cells, any other cell types differentiated or not, known to develop from embryonic stem cells) and to better optimize these conditions. As such, the method of the invention can be used to derive a cell culture with a higher density of desired differentiated cells. The ability to do this may determine whether or not the process is economically viable and therefore is critical to the translation of these technologies from the lab scale.

15 The method of the invention can also be used to identify factors (e.g., any variant, such as growth factors, differentiating factors, such as cytokines, or other conditions, such as pH, temperature, oxygen or factor concentration levels) and conditions that effect and preferably optimize spheroid-forming cell or ES cell differentiation and spheroid or EB formation. Examples of some factors, especially for studying spheroid-forming cell or ES cell differentiation to cardiomyocytes include but are not limited to fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), endothelin-1(ET-1), stem cell factor (SF), opiod peptide (or supplemental DMSO), bone morphogenic protein (BMP) family members transforming growth factor beta (TGF-beta), retinoic acid (RA). Factors preferably used to study beta-cell differentiation include but are not limited to, glucose levels, nicotinamide, KGF (karatinocyte growth factor), EGF (epithelial growth factor, NGF (neural growth factor) and TGF-beta, the above factors, as well as others typically used on adult hematopoietic stem cells (Zandstra et al 1997) can also be used to elicit the development of hematopoietic and endothelial cells from pluripotent cells. This method would comprise culturing the spheroid-forming cells or ES cells under conditions that

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control cell aggregation in the presence of the particular factor to be tested and then detecting the effect of the variant on cell proliferation and differentiation. The effect of various different concentrations or formulations of the factor can also be tested. Preferably a negative control is used, whereby
5 the spheroid-forming cells or ES cells are cultured in the absence of the factor to be tested.

The methods and products of the invention can also be used to produce cells useful for drug therapy testing, to identify targets for gene and cell therapy, and in assays for such methods .

10 Spheroid-forming cells, such as ES cells and the cells and tissues derived therefrom can also be used in the treatment of various cancers, leukemias, autoimmune diseases, organ failure, animal or tissue cloning, gene therapy, transgenic animals.

The spheroid-forming cells and ES cell-derived cells and tissues
15 obtained from the invention can be used to provide cells and tissues for transplantation. For instance, ES cells can be grown under conditions to produce hematopoietic cells. Such cells could be used in bone marrow transplants, blood transfusions or infusions. ES cell derived cardiomyocytes
can be used in tissue engineering, cell/tissue transplantation, gene therapy,
20 and drug discovery. ES cells derived skin tissue can be used for reconstructive surgery, ie., for burn victims, cosmetic surgery to name a few.

Further examples of applications for ES derived cells and tissues can be found in Rathjen et al 1998 or Marshall E. 2000.

In yet another embodiment the invention is directed to a method to
25 identify factors (any substance, media or other conditions, for instance oxygen levels, pH, pressure, cell density) that effect cell proliferation, differentiation and/or spheroid formation or other cellular or spheroid characteristic desired to be monitored, said method comprising culturing the cells as per the method of the invention in the presence of the factor to be tested and then monitoring
30 the effect of the factor on cell proliferation, differentiation and/or spheroid formation or other cell or spheroid characteristic desired. Such monitoring can include comparing the effect of the factor on cells in light of a control culture. In one embodiment, such a control culture can be a negative control

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which does not contain the factor to be tested. Such factors can include factors for screening compounds that may be useful for the treatment or diagnosis of various medical conditions.

It should be noted that although the discussion above describes the invention in terms of ES cells, the same methods and applications and products of the invention would be applicable to pluripotent cells in general or spheroid-forming cells other than ES cells.

The present invention also includes the Spheroid-forming and Embryonic stem cell derived cell culture obtained using the method of the invention, including the initial encapsulated, and/or high density cell culture

In another embodiment the invention provides a method to prevent aggregation between cells comprising encapsulating a cell or group of cells and thus preventing aggregation of said cells with cells not within said capsule. Further the invention also encompassed the use of encapsulating cells, as described herein to control the micro environment of a cell to be cultured. This is not limited to spheroid-forming or ES cells. Such methods can be used to proliferate such cells or to control culturing conditions. To optimize cell culture conditions and to identify factors that effect culture conditions. A liquid suspension comprising encapsulated cells, such as spheroid-forming cells and derived cultures there from are also encompassed within the scope of the present invention.

The following non-limiting examples are illustrative of the present invention:

25 **EXAMPLES**

Materials and Methods

ES Cell Culture

Murine embryonic stem cells: CCE, R1, D3 (mentioned below) and others (Beddington and Robertson, 1989; Nagy, Rossant et al., 1993), were maintained at 37°C in humidified air with 5% CO₂ in Iscoves Modified Dulbecco's Medium (IMDM, GIBCO-BRL) supplemented with 15% fetal bovine serum (Hyclone), 50 U/ml penicillin (GIBCO-BRL), 50 µg streptomycin

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(GIBCO-BRL), 2mM L-glutamine (GIBCO-BRL), 0.1 mM 2-mercaptoethanol, and 500 pM leukemia inhibitory factor (LIF) (ES cell media). Culture flasks were prepared prior to cell seeding by coating with a solution of 0.1% porcine gelatin in phosphate buffered saline. All ES cells were used between passages 15-24.

ES cell differentiation systems

ES cells were dissociated using 0.25% trypsin-EDTA (Sigma) and cultured in ES cell medium without LIF to induce differentiation. Liquid suspension cultures (LSC) were prepared by aliquoting 10 ml of cell solution at the indicated ES cell density into 10 cm petri-dishes (Fisher). For single ES cell analysis (single cell liquid suspension culture scLSC), a single ES cell was placed into each well of a 96 well plate (Nunc) coated with 10% pluronic (F-127, Sigma) solution to prevent cell attachment. This was achieved by preparing a suspension of 20 ES cells/ml and aliquoting 50 μ L into each well. Each well was then visually inspected for the actual number of deposited cells. Empty wells or wells with more than one cell were marked and not included in the analysis. Methylcellulose cultures (MC) were prepared by aliquoting 1 ml of cell suspension with 1% methylcellulose (M3220, Stem Cell Technologies) at the indicated ES cell density in 35mm Greiner dishes (Greiner Labortechnik). Hanging drop cultures (HD) were prepared using 10 μ L droplets, each containing the number of ES cells desired to initiate the EB (as indicated). ES cells were allowed to aggregate in hanging drops for two days before transfer to liquid suspension culture.

Stirred cultures were performed in spinner cultures (Bellco Glass) ranging from 50 to 250ml of media/vessel. ES cells were added to the media directly (stirred cultures without encapsulation: SC) or encapsulated (ESC) as indicated. A paddle-style impeller was used to agitate the media at 40-60 RPM.

EBs were enumerated by visual inspection. EBs were dissociated by incubation (1 min, 37°C) in 0.25% trypsin-EDTA (Sigma), or incubation (30 min, 37°C) in 0.25% collagenase (Sigma) in PBS supplemented with 20% fetal bovine serum for post day 7 EBs. Mechanical shearing was also used to

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help dissociate cells into a single cell suspension by twice passaging the cell mixture through a 21-gauge needle and syringe (Becton Dickinson). Individual cells were then counted using a hemocytometer and analysed by either myeloid-erythroid colony-forming cell (CFC) assay (Zandstra et al. 1997) or flow cytometry (Zandstra et al. 1997)

Gel Microdrop (GMD) Encapsulation of Individual ES Cells

Individual CCE ES cells were suspended in Hanks Buffered Saline Solution (HBSS) at a concentration of 10^7 cells/ml and added to a 3% agarose solution for a final cell concentration of 2×10^6 cells/ml. The agarose solution was dispensed into mineral oil (nonsolvent) at 37°C and subjected to impeller blending using the One Cell System (Ryan et al., 1995) to create agarose microdrops containing individual ES cells. The GMDs were washed twice with HBSS to remove the mineral oil before resuspension in ES cell media without LIF for differentiation culture.

Gel Microdrop(GMD) Encapsulation of Multiple ES Cells

Three different methods were used to encapsulate multiple ES cells.

(1) ES cells were gently dissociated from adhered culture using trypsin, creating multi-ES cell clumps or aggregates. Clumps were subsequently encapsulated as described for individual ES cells.

(2) ES cells were dissociated to a single cell suspension and cultured at high cell density ($>10^5$ cells/ml) in unagitated petri dishes for 12-24 hours to induce cell clump formation via controlled aggregation. The average number of ES cells per clump was controlled by input cell density and by time, and was determined by dividing the input number of ES cells by the number of clumps counted. ES cell clumps were then encapsulated as described.

(3) Individual ES cells were mixed with agarose at a concentration of $1-5 \times 10^7$ cells/ml and encapsulated as described. Capsules formed in this way were essentially clumps of individual ES cell held together by agarose matrix. Capsules themselves were considered ES cell clumps and again encapsulated as described.

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Hematopoietic Colony Forming Cell Assay

Cells were plated into methylcellulose medium (M3434, Stem Cell Technologies) containing 50 ng/ml mrSCF, 10 ng/ml mrIL-3, 10 ng/ml hrIL-6, 3 U/ml hEpo, 15% fetal calf serum, 0.1 mM 2-mercaptoethanol, and 2mM L-glutamine. Hematopoietic colonies were scored by morphology after 6-7 days.

Generation of cardiac myocytes from ES cells

10 CCE and R1 embryonic stem cell were transfected with the MHC-neo^r/PGK-hygro^r DNA as previously described (Klug et al 1996, WO 95/14079, 26 May 1995; U.S. Patent Nos. 5,733,727 and 6,015,671). Briefly, 1 µg of DNA and 25 µg of salmon testes DNA were mixed in a total volume of 800 µl of cells suspended at 4×10^6 cells / mL on ice. Cells were
15 electroporated (180 volts, 80 mF), incubated on ice for 15 minutes, and plated in 100 mm corning dishes in ES media containing 500pM LIF. Cells containing the plasmid were selected by culture in ES medium with LIF and 200 µg/mL hygromycin B for 7 days with daily medium exchange. Test ES cells were then encapsulated using the multiple ES cell encapsulation
20 technique as described above in the third technique. After 8-10 days of culture in stirred suspension bioreactors (Bellco spinner flasks or Applikon 1L cell culture bioreactors) in ES cell media without LIF, the G418 was added to the medium to select for the cardiac cells. The composition of the selected cells was analyzed by MF-20 staining using flow cytometry. 5×10^5 - 10^6 cells to be
25 stained for MF-20 were washed with PBS and fixed with 100 µl of IntraPrepTM Permeabilization Reagent 1 (Immunotech, Westbrook, ME) for 15 minutes at room temperature. After washing once with 1 ml PBS, the cells were permeabilized for 5 minutes with IntraPrepTM Permeabilization Reagent 2 and incubated with a 1:10 dilution of the mouse anti-sarcomeric myosin heavy
30 chain (MF-20) antibody (Transduction Lab, MA) for 15 minutes at room temperature. After washing once with PBS, secondary FITC-conjugated goat anti-mouse IgG antibody was added with 1:100 dilution and the cells were incubated for 15 min at room temperature. In addition, some of the dispersed

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cells were used for determining the viability of the cells immediately after the procedure of enzymatic dissociation. Test cell populations were washed in ice-cold Hanks hepes-buffered salt solution containing 2% FCS (HF) and resuspended at 10^6 cells/100 μ L in HF. Finally, the cells were analyzed on a flow cytometer (XL, Beckman-Coulter, Miami, FL) using the EXpoADCXL4 software (Beckman-Coulter). Positive staining was defined as the emission of a level of fluorescence that exceeded levels obtained by >99.9% of cells from the control population stained with only the secondary antibody. Cardiac development was also determined by electron microscopy.

Generation of Hematopoietic and Endothelial Cells

Hematopoietic and endothelial cells were generated in through ES cell differentiation in ES cell media without LIF under the conditions described in Dang et al. 2002. Input ES cells were encapsulated using the multiple ES cell encapsulation technique as described above in the third technique.

Flow Cytometry for analysis of endothelial and hematopoietic cells

Cells were washed in ice cold Hanks hepes-buffered salt solution containing 2% FCS (HF) and resuspended for 10 min at 4°C in HF containing α -mouse CD16/CD32 monoclonal antibody (PharMingen) at 1 μ g/100 μ L to block non-specific binding. Blocked cells were then incubated at 10^7 cells/ml for 40 min at 4°C with FITC anti-mouse CD34 (PharMingen), PE anti-mouse Flk1 (PharMingen), and anti-mouse E-Cadherin (Sigma). Anti-mouse E-Cadherin incubation was followed by two washes in ice cold HF and then incubated for 40 min at 4°C with anti-mouse FITC-conjugated IgG (Sigma). Stained cells were washed twice in ice cold HF, and 1 μ g/ml 7-amino-actinomycin D (7AAD, Molecular Probes) was added to the final wash. The cells were analyzed on Coulter Epics XL using 4-Colour Expo 32 software for analysis. Positive staining was defined as the emission of a level of fluorescence that exceeded levels obtained by >99.9% of the cells from the same starting population stained with a matched fluorochrome-labelled irrelevant isotype control antibody.

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Measurement of Media composition

Oxygen tension, in addition to other parameters, was measured by withdrawing 3ml of culture media using a gastight syringe (Hamilton), and analyzing using a BioProfile 200 blood/gas analyzer (Nova Biomedical).

5

Neurosphere formation

R1 murine embryonic stem cells were dissociated into a single cell suspension and transferred at 10^4 ES cells/ml to media comprised of Dulbecco's Modified Eagle Medium (DMEM, GIBCO-BRL) supplemented with 20% Knockout serum replacement (Sigma), 50 U/ml penicillin (GIBCO-BRL), 50 µg streptomycin (GIBCO-BRL), 2mM L-glutamine (GIBCO-BRL), and 0.1 mM 2-mercaptoethanol.

10

Example 1 - Effects of Cell-Cell Adhesion and EB Aggregation on ES cell**culture**

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To assist in the development of a new bioprocess, the properties of ES cells and EBs that might affect bioprocess efficiency, net cell expansion, and cell fate decisions were investigated. EB formation, growth and development between different culture techniques were compared. Once an understanding of these basic parameters was obtained, guidelines to the design of efficient, scalable bioprocesses were developed.

(a) Some ES cell lines can form EBs from individual cells.

25

30

Preliminary studies focused on establishing the *in vitro* developmental potential of individual ES cells. In single cell liquid suspension (scLSC) and methylcellulose culture (MC), EB efficiency (number of EBs divided by input number of ES cells) have been reported anywhere between 2-15%. Because these values were so low, it was uncertain as to whether an individual ES cell could form an EB, or if EB formation occurred only when ES cells aggregated by chance (or by design, as in the hanging drop method). Therefore the first constraint investigated was the number of ES cells required to form an EB.

Individual murine CCE ES cells were placed by limiting dilution into separate wells of a 96 well plate and differentiated in liquid suspension culture

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(ES media without LIF). One hour after plating, each well was visually examined to verify the number of cells plated. After seven days of differentiation, the wells were examined for cell aggregates. The cell aggregates were identified as EBs based on their expression of Flk-1 and CD34, and their ability to form hematopoietic colonies (data not shown). By dividing the total number of EBs per 96 well plate by the number of input ES cells, we determined that individual ES cells were able to form EBs with an overall efficiency of at least $42 \pm 9\%$ (Table 1). In a separate set of experiments, it was determined that $78 \pm 9\%$ of the starting cell population were undifferentiated ES cells based on E-cadherin and SSEA-1 expression (data not shown, (Zandstra, et al. 2000)). Viability, based on exclusion of dead cell dye 7AAD, of the ES cell population after trypsinization was determined to be $87 \pm 11\%$. This allowed recalculation of the EB formation efficiency from individual, undifferentiated, and viable ES cells to be approximately 60%. These results clearly demonstrated that a majority of individual murine CCE ES cells have the capacity to form an EB, and that these cells could form EBs at a very high efficiency. However, the results also raise the issue of why such high efficiencies were not regularly achieved in standard differentiation cultures.

The present inventors have shown that murine CCE ES cells have demonstrated the ability to form EBs from single ES cells. In other cell types or under other conditions contact between multiple ES cells to enable EB formation may be preferred. For example, the present study repeated with single murine R1 and D3 ES cells did not form single ES cell derived EBs under the conditions used. Instead, aggregation with approximately 5 or more ES cells was required for survival, proliferation and differentiation (see below). Similarly, it has been reported [Thomson JA, Odorico JS. 2000] that current human ES cell lines form EBs only from multi-ES cell aggregates or clumps. Regardless, the following aggregation studies reported with CCE ES cells are still illustrative of the extent of EB aggregation and the problem it poses. As such controlling cellular aggregation during expansion and differentiation of said cells is an important factor.

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(b) EBs aggregate, reducing EB formation efficiency

Further investigation revealed the sensitivity of EB efficiency to initial ES cell concentration and time (Figure 3). Various initial CCE ES cell concentrations were differentiated in liquid suspension and methylcellulose cultures, and the number of cell aggregates (EBs) per dish was recorded at regular time intervals. In liquid suspension culture, EB formation efficiency increased as ES cell concentration decreased (Figure 3A). Over time, EB formation efficiency decreased for all cell concentrations. However the extent of this decrease was most dramatic in cultures initiated with 10^5 ES cells/ml, and almost nonexistent at 10^2 ES cells/ml. In methylcellulose cultures (Figure 3B) initiated with 10^3 ES cells/ml or less, EB formation efficiency remained constant at approximately 40% over the duration of the experiment. EB formation efficiency gradually decreased in methylcellulose cultures initiated with 10^4 or more ES cells/ml, however to a lesser extent than their liquid suspension counterparts.

Based on their similarity, the model of the present invention for EB formation from single ES cells ($42 \pm 9\%$) was reconciled with EB efficiency in methylcellulose culture initiated with 10^3 ES cells/ml or less ($40\% \pm 6\%$) and liquid suspension culture initiated with 10^2 ES cells/ml or less ($34 \pm 5\%$). However, an explanation was still required for the discrepancy with the results obtained at higher cell concentrations. It was recognized that the decrease in EB efficiency over time indicated that the process of EB formation itself was unaffected; rather, another process occurring post-EB formation must be reducing the number of EBs present. Observing the cultures, it was apparent that EB aggregation was the cause of inefficiency. In this process, separate EBs aggregated and merged into a single, larger EB.

This phenomenon accounts for decreased EB efficiency with increased cell concentration since likelihood of EB collision and subsequent aggregation would also be greater. Furthermore, aggregation explains the higher EB efficiency in methylcellulose cultures compared to liquid suspension culture: Semi-solid methylcellulose media impaired cell movement, thereby decreasing likelihood of EB collision and aggregation.

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(c) Aggregation reduces net cell expansion

The results demonstrate that CCE ES cells form EBs with an efficiency of $42 \pm 9\%$, a value independent of culture method and initial ES cell concentration, and that EB aggregation reduced the apparent efficiency over time. To determine what effect EB aggregation might have on the overall production of cells, the growth rate of various initial ES cell aggregate sizes (1, 100, and 1000 ES cells/EB) formed using the single cell liquid suspension culture (scLSC) and hanging drop techniques were compared (Figure 4, Table 2). Regardless of initial aggregate size, all EBs contained a similar number of cells by day 12. Since final EB size is independent of EB aggregation, EB aggregation reduces overall expansion of cells.

(d) Cell-cell adhesion molecules are responsible for EB aggregation

The process of EB aggregation was studied using the simplest scenario: aggregation of two EBs. To visualize cell mixing, one EB generated from wildtype ES cells was aggregated with one EB generated from ES cells constitutively expressing GFP (Hadjantonakis et al 1998) . The extent of aggregation was quantitatively determined by the "degree of aggregation" (DOA) - the ratio of the diameter of the interface to the overall length of the two EB system (Figure 5). Regardless of initial aggregate size (50, 100, 400 ES cells/EB, data not shown), complete fusion (DOA > 90%) was achieved after approximately 16 hours and complete cell mixing (homogenous fluorescence intensity) after 48 hours (Figure 5).

The mechanism of EB aggregation was investigated using various loss of function treatments (Figure 6). Blocking surface cell adhesion molecules with blocking peptide inhibited EB aggregation. E-cadherin blocking was particularly efficient, also noted by Larue et al. (1996) who observed that homozygous E-cadherin null ES cells were unable to aggregate as clumps. EBs treated with cytochalasin D also were unable to aggregate. Cytochalasin D inhibits actin-dependent processes including cell migration and cell division. To be sure that impaired cell division was not responsible for this effect, another group was treated with Mitomycin C that inhibited mitosis by preventing DNA synthesis. These EBs aggregated at the same rate as the

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control (no treatment). Based on these results, a two-step mechanism for EB aggregation was proposed: First, neighboring EBs collide and homophilic E-cadherin molecules adhere, holding the EBs together. Cells then migrate and remodel as the EBs fuse into a single spheroid.

5 Preventing cell adhesion versus cell migration between neighboring EBs was the logical strategy for preventing EB aggregation as treatments that could affect cell growth or differentiation (i.e. cytochalasin D) would be undesirable for most applications. E-cadherin, the prominent cell adhesion molecule in this process, is a Ca^{2+} -dependent homophylic cell-cell adhesion
10 molecule expressed by ES cells. As ES cells differentiate, E-cadherin expression is downregulated (Zandstra et al. 2000). Using flow cytometry, E-cadherin expression was tracked in differentiating EBs over time (Figure 7.A). Expression decreased as cells differentiated - from almost 100% on day 0 (ES cells) to approximately 25% by day 5 of differentiation. As expected, E-
15 cadherin expression correlated with rate of EB aggregation: EBs differentiated for four days or fewer aggregated within 16 hours, while day 6 EBs did not aggregate (Figure 7.B). Similarly, while ES cells placed into stirred culture quickly aggregated into large cell clumps that did not grow or differentiate properly, EBs grown in static culture for a minimum of four days could be
20 transferred to stirred culture with little aggregation. These EBs continued to grow and differentiate normally. Therefore, the strategy adopted for controlling cell adhesion need only be implemented while cell adhesion molecule expression remains high. For murine ES cells, this time period is the first four days of differentiation.

25 These results suggest that novel approaches to control the aggregation of undifferentiated ES cells and their derivatives may allow them to be cultured in high cell density, stirred suspension bioreactors.

Example 2 - Preventing ES Cell-Cell Adhesion and EB Aggregation **Results in Efficient Generation of Embryonic Stem Cell Derived** **Hematopoietic and endothelial cells**

30

The overall schematic of one embodiment of the bioprocess of the invention for the scalable culture of ES cell derived tissues is shown in Figure

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2. First, undifferentiated ES cells are expanded under appropriate conditions. ES cells are then cultured in a bioreactor conditions that control aggregation and promote cell proliferation and differentiation. More specifically, aggregation of ES cells to induce EB formation is permitted and aggregation
5 between cells within an EB is permitted. However aggregation between ES cells beyond that required to induce EB formation is prevented, and aggregation between separate EBs is prevented.

After an appropriate period of culture, under defined conditions (including growth factor and medium formulation, oxygen tension, pH, medium
10 supplementation rates), differentiated cells are selected for and used in clinical or pharmaceutical applications. In one aspect, the present invention is primarily concerned the scalable generation of ES cell derived cells and tissues.

15 **(a) Culture of EB Under Non-Aggregating Conditions**

Genetically modified ES cells

E-cadherin⁻ ES cells are defective in cell adhesion, unable to grow as clumps or aggregates of cells as they typically do (Larue, Antos et al. 1996).

20 *Antibodies to block cell adhesion molecules*

As reported in the present study of EB aggregation, treatment of EBs with E-cadherin blocking peptides inhibits EB aggregation. Blocking peptides effectively nullifies the function of E-cadherin, as reported by Burdsal et al. (1993). Blocking E-cadherin prevents EBs from adhering with neighboring
25 EBs, thus circumventing subsequent aggregation.

Reducing inorganic salt concentration or inhibition of ion channels

Cell adhesion can be inhibited by substantially reducing inorganic salt concentration (Boraston et al. 1992). For example, cell adhesion modulated
30 by calcium-dependent adhesion molecules such as cadherins depends upon calcium influx from both extracellular calcium through calcium channels and release from internal calcium stores. Calcium channel inhibitor LaCl₃ or thapsigargin was shown to inhibit cell-cell attachment (Ko, Arora et al. 2001).

(b) Encapsulation of ES cells

Preventing EB aggregation was the guiding design principle for new bioprocesses capable of efficient and scalable differentiation of ES cells.

- 5 While it was suspected that E-cadherin was the main molecule involved in cell aggregation, a method was developed to preclude interactions between all cell-cell adhesion molecules on the EB surface by physically blocking their association. In this method, ES cells are encapsulated within a biodegradable matrix and allowed to grow and differentiate in liquid culture, such that each
- 10 cell-containing capsule gives rise to a single EB. The surrounding matrix prevents separate EBs from contacting one another, thereby preventing aggregation. Formation of EBs in this novel way permits high cell density culture in a homogeneous, controllable, and scalable bioreactor system. The bioprocess of the present invention is termed: encapsulated stirred culture
- 15 (ESC). The first step in this method involves the encapsulation of individual or multiple ES cells. Uniform or "synchronized" EB sizes could be achieved by encapsulating similar numbers of ES cells per capsule. The matrix could be agarose, alginate, or another type of natural or artificial matrix (e.g. polymer) that does not interfere with the growth and differentiation of EBs. Depending
- 20 upon the matrix used, cells might degrade the matrix as they grow (as is the case with agarose), or they might burst the capsule as cell growth places increasing pressure on the capsule (as is the case with alginate), or they can be manually released from their capsules by adding degradation enzymes (such as agarase addition to agarose), or changing the culture conditions
- 25 (such as depleting dissolved calcium ions in the case with alginate). Therefore, the size and composition of the capsule can determine the amount of time the EB remains encapsulated. For example, agarose is a simple polysaccharide readily degraded by the cells as they grow. Therefore, the diameter of the agarose capsules determines the amount of time the EB
- 30 remains encapsulated (Figure 8.A, Table 3). Capsules are designed to retain the EB until cell differentiation results in downregulation of E-cadherin (or other cell adhesion molecule) expression. After this time, EBs that emerge from the capsules will not aggregate. For differentiating EBs from murine ES

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cells, E-cadherin is sharply down regulated after 4 days (Figure 7.A). It was determined that 70-100 μm agarose capsule diameters provide adequate protection for this period of time, and such capsules were specifically designed (Figure 8.B). However, it should be understood that the invention is not intended to be limited to such a capsule size as a person skilled in the art would appreciate that other sizes would work.

(c) Encapsulation enables use of stirred culture

ES cells placed directly into stirred culture quickly aggregate into large cell clumps that did not proliferate or differentiate into any of the cell types assayed for (data not shown). Encapsulating ES cells prior to their introduction to stirred culture enabled efficient EB formation and prevented their aggregation. EB efficiency between liquid suspension culture (LSC) [non-stirred, non-encapsulated], stirred culture (SC), and encapsulated stirred culture (ESC) was compared at 10^3 and 10^4 input ES cells/ml after 7 days of differentiation culture (Table 4). Whereas EB efficiency in LSC declined as ES cell density increased, EB efficiency remained high in ESC.

Fold expansion of total cells in liquid culture (LSC), stirred culture (SC), and encapsulated stirred culture (ESC) is illustrated in Table 5. Cultures were initiated with 10^4 R1 ES cells/ml and analyzed after 7 days of differentiation. Table 5 shows results of fold expansion, after adjusting for cell loss during encapsulation procedure. Yield of encapsulated ES cells was approximately 25% of input. Cells were lost during the various transferring stages between centrifuge tubes, or retained in the mineral oil phase.

In stirred culture, EB efficiency was significantly improved with encapsulation than without. Encapsulated ES cells were able to form EBs that remained shielded from one another, thus maintaining a high EB efficiency by preventing EB aggregation. EBs emerged from the capsules after approximately four days - coinciding with down regulation of E-cadherin as previously described. Emerged EBs did not aggregate.

The ability to use stirred or other well-mixed systems ensure that bulk media conditions are homogenous, thus facilitating measurement and control

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of the system. These culture systems can also accommodate increase demand in cell generation by simply increasing the volume of the tank.

(d) Bioreactor does not significantly affect cell growth or differentiation

5 Hematopoietic development within EBs has been extensively studied and well characterized (Keller 1995). Hematopoietic development was therefore selected as the measure by which ES cell differentiation in different culture systems was compared. ES cells become blood cells through a series of developmental steps that can be tracked by cell surface marker expression
10 (Table 6). Murine ES cells differentiate into Flk-1 expressing mesoderm on day 4 that subsequently give rise to CD34 and CD45 expressing hematopoietic progenitor cells. Hematopoietic progenitors can also be assayed for using the hematopoietic colony forming cell (CFC) assay.

Ideally, the encapsulating matrix physically prevents EB aggregation
15 but does not impede transport of nutrients and metabolic products to and from the cells, nor influence differentiation or growth of EBs in any manner. To demonstrate this, ES cells were encapsulated in agarose and differentiated in static liquid culture. Cell proliferation and differentiation in this system was compared to standard ES cell differentiation systems: liquid suspension
20 culture, hanging drop, and methylcellulose cultures. Therefore agarose encapsulation of ES cells did not affect EB cell growth in any significant way.

Cell proliferation and differentiation was also compared between liquid culture (LSC) and the bioprocess enabled by the invention: encapsulated stirred culture (ESC) (Table 7). ESC was able to generate the hematopoietic
25 cell types following the same developmental kinetics as LSC. Cell proliferation and hematopoietic cell frequencies were comparable but not necessarily equivalent between the two systems.

EXAMPLE 3 - Use of Bioprocess to Generate Differentiated Cell Types

30 Generation of various differentiated cell populations was performed using encapsulated ES cells grown in stirred suspension bioreactors compared to LSC.

a) Generation of hematopoietic cells from ES cells.

Mentioned previously, early mesoderm development was tracked by Flk-1 cell surface marker expression on day 4, while hematopoietic progenitors were detected phenotypically by co-expression of CD34 and CD45 cell surface markers, or functionally via methylcellulose colony assays. Using our novel invention, various types of hematopoietic progenitor cells were detected, from multipotent myeloid cells (CFC-Mix), to further restricted myeloid and erythroid cells (CFC-Ep, CFC-Ed, and CFC-GM) (Table 7). ES cells were differentiated in ES cell media in the absence of LIF. Because the culture was performed in a stirred bioreactor, it is feasibly scalable to any desired volume, and applicable to close microenvironmental control

b) Generation of endothelial cells from ES cells.

Using the same approach as described above (ES cell differentiated in ES cell media in the absence of LIF) in section a), endothelial cells were detected by coexpression of multiple endothelial cell markers: Flk-1, CD34, and CD31. A gate was drawn around Flk-1 and/or CD34 expressing cells, enabling exclusive analysis of gated cells for expression of CD31 (Figure 9). Because the culture was performed in a stirred bioreactor, it is feasibly scalable to any desired volume, and applicable to close microenvironmental control.

c) Generation of other cell types from ES cells

Other researchers have demonstrated that other cell lineages can also be generated from ES cells *in vitro*, including hepatocytes, β -cells, neurons, glial cells, kidney cells, muscle cells, endothelial cells, hematopoietic cells [i.e., Wang et al, 1992 for endothelial cells; Rohwedel et al., 1994, for cardiac and muscle cells 1994; Bain et al, 1995 for neuronal cells; Palacios et al, 1995 for hematopoietic stem cells; Choi et al, 1998 for hematopoietic and endothelial cells; Fleishman et al, 1998 for cardiac cells; Tropepe et al, 1999 for neural cells], and other cell types known to be derived from ES cells in the art. These cell types can be generated in stirred or mixed bioreactor cultures using media and general environment conditions as described in the prior art,

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only when aggregation is controlled, such as is possible using the bioprocess of this invention. This would be so as it was demonstrated that the bioprocess of the invention, e.g., encapsulation, does not adversely affect ES cell differentiation. The fact that this technology of the invention allows for the normal differentiation of ES cells, while facilitating the large-scale production of ES cell-derivatives makes it useful for a variety of cellular therapy-related applications.

Example 4 - The system facilitates measurement and control of culture environment.

Current ES cell differentiation processes are not amenable to measurement and control because the static and batch-style nature of these systems result in spatial and temporal gradients. In well-mixed (dynamic) systems, the bulk media conditions are the same everywhere at any given time. That is, any point measurement made within the system would be reflective of any other point. In this way, one can easily and accurately measure bulk conditions and also introduce control elements.

Using the bioreactor system of Example 2 above, EB development in normoxic (>160 mmHg O_2 tension) and hypoxic (30-40 mmHg O_2 tension) conditions was compared. Oxygen tension was measured offline using a blood gas analyzer and gas mixture in the incubator headspace was adjusting accordingly. Hematopoietic cell frequency, determined by CD34 and CD45 expression and hematopoietic CFC assay, was higher for EBs generated in hypoxic conditions (Table 8), indicating the positive effect that the ability to control and manipulate the microenvironment, such as allowed by this invention will have on the generation of cells and tissue from pluripotent cells.

The tendency for ES cells and early EBs to aggregate prevents the use of well-mixed bioreactor systems as well as higher cell density cultures. Therefore current differentiation systems are limited to static and batch-style conditions. The bioprocess of the invention enables ES cells to efficiently form EBs in well-mixed culture by controlling aggregation. As mentioned, the use of well-mixed systems automatically permits bulk media measurements and implementation of control strategies.

Example 5 – The Generation of Embryonic Stem Cell Derived Cardiac cells using ES cells Transfected with selectable markers

To demonstrate the improved ability to generate large numbers of clinically relevant cell types, the bioreactor culture of encapsulated ES cells was used to generate and select for cardiac myocytes. As for the above hematopoietic and endothelial development, flow cytometric analysis was employed to quantitatively evaluate the percentage of ES cell-derived cardiomyocytes generated during the course of differentiation in stirred bioreactors. In this case, ES cells transfected with the selection cassette as outlined above and described in detail in (Klug et al 1996, WO 95/14079, 26 May 1995; U.S. Patent Nos. 5,733,727 and 6,015,671) were cultured for 9 days in 500 mL stirred suspension bioreactors, prior to the addition of G418 to the media. At given time points the EBs were dissociated, the procedure previously described, resulting in a single cell suspension to be stained with a monoclonal antibody specific for sarcomeric myosin heavy chain (MF-20).

Figure 10 Panel A shows kinetic changes of the percentage of the MF-20 positive cells during the course of differentiation and selection with G418 of transgene – containing ES cells cultured in stirred suspension bioreactors. Figure 10 panel B shows morphologic and structural analysis of bioreactor – grown ES cell-derived cardiomyocytes. The cells exhibit a mature sarcomeric organization and Z-banding (arrow). Figure 10 panel C shows a cross section of a “cardic body” (a spheroid of ES cell derived cardiac cells) generated in bioreactors.

Results showed that only a trace amount of MF-20 staining-positive cells was present in the EBs (0.7%) on day 0 after differentiation, however, the percentage of cells displaying positive staining with MF-20 rose gradually as a function of time after selection by G418, and reached over 70% at day 20 after differentiation. In contrast, the expression level of Oct-4, which is expressed in embryonic stem cells and is one of the first transcription factors differentially regulated during mouse development (Nichols and Zevnik, 1998) decreased significantly during the time course of differentiation, from 68.5% at day 0 to undetectable levels (at day 20 after differentiation (data not shown).

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The observation indicated that at the late state of differentiation and G418 selection, Oct4 positive ES cells were eliminated from the culture system. It is noteworthy that by day 15, the bioreactors typically contained between 2.5×10^7 to 1×10^8 viable cardiac myocytes (as assessed by MF20 expression).

- 5 These observations were consistent in three independent experiments. In some experiments chemical factors such as retinoic acid were added to the media at specific time points to enhance cardiac differentiation and reduce the presence of undifferentiated ES cells (data not shown). The results indicate that differentiation of ES cells with the G418-selection system in spinner flask
- 10 suspension culture system is capable of producing large quantity of cardiomyocytes.

Example 6: Generation of chimeric spheroids containing predetermined types of cells.

- 15 To show that the encapsulation system was capable of controlling the types of cells present in the spheroids at input, two populations of R1 ES cells, each labeled with a specific fluorochemical (green fluorescent protein, GFP) and (cyan) were encapsulated (see Hadjantonakis et al. 1998 and Hadjantonakis AK, Nagy 2001 for descriptions of the cells lines).

20 **Materials and Methods**

- Individual R1 ES cells expressing the GFP protein by a constitutively active promoter (Hadjantonakis et al 1998) and individual trophoblast stem cells labelled with cyan as previously described (Tanaka et al. 1998) were suspended in Hanks Buffered Saline Solution (HBSS) at a ratio of 1 ES cell to
- 25 1 TS cell at a total cell concentration of 2×10^7 cells/ml and added to a 3% agarose solution for a final cell concentration of 4×10^6 cells/ml. The agarose solution was dispensed into mineral oil (nonsolvent) at 37°C and subjected to impeller blending using the One Cell System (Ryan et al., 1995). Microdrops containing a single ES cell and a single TS cell were identified and isolated by
- 30 their side scatter characteristics, and by their GFP and cyan fluorescence using a FACSVantage Flow Cytometer (Becton Dickinson). Isolated microdrops were put sorted into ES cell media without LIF for analysis of their growth characteristics.

Example

To show that the encapsulation system was capable of controlling the types of cells present in the spheroids at input, ES cells and TS cells, each labelled with a specific fluoro-chrome (green fluorescent protein, GFP) and cyan ^ labelled cells) were sorted into 96 well plates. Microscope based tracking of the cells confirmed that the two cell types survived and proliferated and formed spheroids containing two distinct sources of cells (chimeric spheroids). This technology provides the capacity to combine, in spheroids, two or more types of cells, including pluripotent cells and differentiated cells, for the end goal of manipulating the differentiation of the pluripotent cells into specific types of tissue using cell specific signals.

Example 7: Formation of ES cell derived neurospheres

ES cells were differentiated in serum-free conditions, yielding spheroids that did not stain for mesoderm markers. ES cells cultured in chemically defined serum-free, feeder layer-free, low-density culture conditions readily acquire a neural identity (Tropepe et al. 1999). Under these conditions, ES cells transition to a spheroid-forming primitive neural stem cell population capable of self-renewal and neural and non-neural potential - analogous to the neurosphere forming neural stem cells reported by Bjornson et al. (1999).

SUMMARY

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims. For instance, although ES cells were used in the Examples, the invention can be applied to other types of spheroid-forming or pluripotent cell types, such as embryonic germ cells and early primitive ectoderm-like cells or other type of

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stem cells such as neuronal stem cells or cells that can differentiate into other cell types and form spheroids *in vitro* during the differentiation process. Controlling aggregation during any type of cell expansion can also be beneficial in improving expansion efficiency of said cell type.

5. All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

TABLE 1

- EB formation efficiency from CCE muES cells. A single ES cell was deposited into each well of a 96 well plate. Wells were visually inspected for
- 5 EBs after 7 days. EB formation efficiency was determined by the ratio of EBs per 96 well plate, to the number of input ES cells per 96 well plate. Percentage of viable ES cells in the culture was determined by E-cadherin expression and exclusion of 7AAD dead cell stain.

Experiment Number	Input # of ES Cells	# of EBs	Actual EB Formation Efficiency	% E-Cad Positive Cells	Viability	% Viable ES Cells	Calculated EB Formation Efficiency
1	86	38	44.2%	68.5%	90%	61.7%	
2	97	33	34.0%	85%	75%	63.8%	
3	68	37	54.4%	80%	96%	76.8%	
4	70	26	37.1%	—	—	—	
Average	80.3	33.5	42%	78%	87%	68%	63%
Standard Deviation	13.8	5.4	9%	9%	11%	14%	17%

TABLE 2

Total cell expansion from EBs initiated with 1, 100, or 1000 cells/EB.
Cell expansion improved significantly as fewer ES cells were used to initiate

5 EB formation on day 0.

Initial Cells/EB (day 0)	Maximum Cells/EB	Expansion
1	17300 \pm 9900	17300
100	35100 \pm 11000	351
1000	35000 \pm 3000	35

TABLE 3

Effects of different encapsulation protocols on capsule diameter. 3
different parameters: impeller speed (revolutions per minute, RPM), time
10 (minutes), and ambient temperature, are used to control capsule diameter.

Protocol step	35 μ m	50 μ m	85 μ m
1	2100 RPM 1 minute Room temp	1500 RPM 1 minute Room temp	1200 RPM 1 minute Room temp
2	2100 RPM 1 minute Ice bath	1500 RPM 1 minute Ice bath	1200 RPM 1 minute Ice bath
3	1100 RPM 10 minutes Ice bath	1100 RPM 5 minutes Ice bath	700 RPM 5 minutes Ice bath

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TABLE 4

EB Efficiency in liquid culture (LSC), stirred culture (SC), and encapsulated stirred culture (ESC). Cultures were initiated with 10^3 or 10^4 CCE ES cells/ml and analyzed after 7 days of differentiation. EB efficiency in LSC declined as cell density increased. Aggregation occurred readily in SC regardless of cell concentration. Encapsulation effectively controlled cell aggregation, maintaining a high EB efficiency in stirred culture.

	LSC	SC	ESC
10^3 ES cell/ml	42±9%	>0.1%	41±21%
10^4 ES cells/ml	8.5±2.7%	>0.1%	35±3%

TABLE 5

Fold expansion of total cells in liquid culture (LSC), stirred culture (SC), and encapsulated stirred culture (ESC). Cultures were initiated with 10^4 R1 ES cells/ml and analyzed after 7 days of differentiation. The table shows results of fold expansion, after adjusting for cell loss during encapsulation procedure. Yield of encapsulated ES cells was approximately 25% of input. Cells were lost during the various transferring stages between centrifuge tubes, or retained in the mineral oil phase.

	LSC	SC	ESC
10^4 ES cells/ml	27±13	> 1	63±33

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TABLE 6**Hematopoietic and endothelial cell surface markers.**

Flk-1	Vascular endothelial growth factor receptor expressed by early mesoderm, vascular progenitor, and endothelial cells.
CD34	Antigen expressed by hematopoietic stem, hematopoietic progenitor, and endothelial cells.
CD45	Antigen expressed by hematopoietic cells
CD31	Cell adhesion molecule expressed by platelets, endothelial cells, and murine ES cells.

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TABLE 7

5 Comparison of cell proliferation and differentiation between standard ES cell differentiation culture: liquid culture (LSC), and bioprocess of the invention: encapsulated stirred culture (ESC). Proliferation reported as total cells per EB. Flk-1 expressing mesoderm with hematopoietic potential was assayed on day 4 of differentiation culture. Hematopoietic progenitors coexpressing CD34 and CD45, and/or capable of hematopoietic colony formation (CFC) were assayed on day 7.

Analysis	LSC	ESC
Day 4 - Mesoderm		
Total Cells/EB	700±300	1200±450
Flk-1 ⁺ cells	44±12%	38±8%
Day 7 - Hematopoietic		
Total Cells/EB	3000±1500	3500±700
CD34 ⁺ CD45 ⁺ cells	1.9±1.2%	1.0±0.6%
CFCs per 10 ⁵ cells	14.0±6.0	5.0±2.0

10

TABLE 8

Comparison of hematopoietic cell development (on day 7 of differentiation culture) between normoxic (>160 mmHg O₂) and hypoxic (30-40 mmHg O₂) encapsulated stirred culture (ESC).

Analysis (Day 7)	Normoxic ESC	Hypoxic ESC
Total CD34 ⁺ cells	6.1%	8.0%
Total CD45 ⁺ cells	0.9%	2.6%
CD34 ⁺ CD45 ⁺ cells	0.6%	1.6%
CFCs per 10 ⁵ cells	5±2.0	7±2.6

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TABLE 9**Cell Lineage-Specific Promoters**

Promoter	Obtained Cell Lineage	Literature
β -cardiac myosin heavy chain promoter	cardiomyocytes	Klug et al. (1996) J. Clin. Invest. 98:216-24
MLC-2v	cardiomyocytes	Muller et al. (2000) FASEB J. 2000 Dec;14(15):2540-8
human atrial natriuretic factor (hANF) promoter	cardiomyocytes	Wu et al. (1989) J. Biol. Chem. 264:6472-79
SOX2 gene	neural precursors	Li et al. (1998) Curr. Biol. 8:971-4
Human insulin promoter (HIP)	Beta cells (insulin-secreting cells)	Soria et al. (2000) Diabetes 49:157
PDX1 promoter	Beta cells (insulin-secreting cells) pancreatic cells	Gannon M. et al. (2001) Dev Biol. 2001 Oct 1;238(1):185-201
Pax4 promoter	Beta cells (insulin-secreting cells)	Brink C. et al. (2001) Mech Dev. 2001 Jan;100(1):37-43, Smith SB. et al. (2000) J Biol Chem. 2000 Nov 24;275(47):36910-9.
VE-Cadherin promoter	Endothelial cells	Gory S. et al. (1999) Blood. 1999 Jan 1;93(1):184-92.
human VE-Cadherin-2 promoter	Endothelial cells	Ludwig D. et al. (2000) Mamm Genome. 2000 Nov;11(11):1030-3

5

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TABLE 10

- 5 Examples of cell types derived from the in vitro differentiation of ES cells, as well as examples of additives thought to encourage the differentiation / survival of the specific cell types are indicated. In most cases the additive is added to serum containing media; examples of cells generated in a Chemically Defined Media (CDM) are also listed.

Cell Type Generated	Example Additives	Reference
Primitive ectoderm	CDM	Tropepe et al. 2001
Mesoderm precursors	BMP4, Activin A	Wiles et al. 1999
Endothelial cells	VEGF	Eichmann et al. 1997
Hematopoietic cells	IL-11+KL, IL-1 VEGF+KL VEGF+BMP4 IL-2+IL-3+Con A	Keller et al. 1993 Kennedy et al. 1997 Nakayama et al. 2000 Chen et al. 1992
Erythroid cells	Epo	Wiles et al. 1991
Myeloid cell	IL-3+IL-1+Epo, IL-3+IL-1+M-CSF / GM-CSF IL-3+M-CSF	Wiles et al. 1991 Lieschke et al. 1995
Lymphoid cells	VEGF+BMP4 RP.0.010+IL3+IL-6+IL-11	Nakayama et al. 2000 Gutierrez-Ramos et al. 1992
Skeletal muscle	TGF β 1 / TGF β c2 DMSO	Slager et al. 1993 Dinsmore et al. 1996
Cardiac muscle	TG β 1 / TGF β 2 RA	Slager et al. 1993 Wobus et al. 1997
Neuroectoderm	CDM NGF bFGF	Tropepe et al. 2001 Yamada et al. 1994 Okabe et al. 1996
Neurons	RA	Bain et al. 1995
Adipocytes	RA+insulin+T3	Dani et al. 1997
Beta-cells	B27 Supplement & bFGF B27 Supplement & Nikotinamid	Lumelsky NO. et al.2001
Beta-cells	Nikotinamid	Soria B. et al. 2000
Beta-cells	EGF, HGF and Nikotinamid	Ramiva VKM. et al. 2000

5

TABLE 11

Examples of cell types derived from the in vitro differentiation of human embryonic stem cells, as well as examples of additives thought to encourage the differentiation / survival of the specific cell types are indicated.

Cell Type Generated	Example Additives	Reference
Mesodermal cells	Activin-A and TGFbeta1	Schuldiner M, et al. 2000
Ectodermal, Mesodermal cells	Retinoic acid (RA), EGF, BMP-4, bFGF	Schuldiner M, et al. 2000
Ectodermal, Mesodermal Endodermal cells	NGF and HGF	Schuldiner M, et al. 2000
Neuronal cells	Retinoic acid (RA), nerve growth factor (betaNGF)	Schuldiner M, et al. 2001
Hepatocytes	sodium butyrate	Rambhatla L, et al. 2001
Beta-cells		Assady S, et al. 2001
Cardiomyocytes		Kehat I, et al. 2001
Endothelial cells		Levenberg S, et al. 2002

**FULL CITATIONS FOR REFERENCES REFERRED TO IN THE
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WHAT IS CLAIMED IS:

1. A method of generating cells derived from spheroid-forming cells comprising culturing spheroid-forming cells under controlled cell aggregation conditions that enable spheroid formation in liquid suspension.
2. The method of claim 1 wherein the conditions enable spheroid formation in liquid suspension under high cell density.
3. The method of claim 1 or 2 wherein the cells are kept in liquid suspension by stirring.
4. The method of any one of claims 1-3 wherein controlled cell aggregation conditions means preventing spheroid aggregation.
5. The method of any one of claims 1- 4 wherein controlled cell aggregation conditions means permitting cell aggregation sufficient for spheroid formation and preventing aggregation between cells of different spheroids.
6. The method of claim 4 or 5 wherein the preventing aggregation is done by any one or more of the following means:
 - (a) preventing physical association between cell aggregation molecules;
 - (b) removing inorganic salts from the cell culture;
 - (c) blocking surface aggregation molecules with specific peptides;
 - (d) using spheroid-forming cells that are cell adhesion molecule-null spheroid-forming cells; and
 - (e) inhibiting cell adhesion molecule expression and/or activity.
- (f) Methods that prevent cell aggregation by physically separating one or more spheroid-forming cells to enable spheroid formation in distinct compartments such as described in the single cell liquid suspension culture (scLSC) method in this patent without limitation to a single cell.
7. The method of any one of claims 1-6 wherein the controlled aggregation is affected by encapsulation of spheroid-forming cells .
8. The method of claim 7 wherein each capsule contains an initial number of spheroid-forming cells sufficient to enable spheroid formation.
9. The method of claim 8 wherein one spheroid is formed per capsule.

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10. The method of claim 8 wherein the initial number of cells is 1.
11. The method of claim 7 wherein the capsule initially comprises multiple cells of at least two different cell types.
12. The method of claim 11 wherein a spheroid is formed and the spheroid is a chimeric spheroid.
13. The method of claim 7 wherein the capsules have a capsule body comprising a matrix that permits passage of desired media constituents through the matrix to the spheroid-forming cells and spheroid, but do not permit aggregation with cells outside the capsule.
14. The method of 7 wherein the gel microdrop method is used to encapsulate the spheroid-forming cells.
15. The method of any one of claims 1-14 wherein the cells are cultured under conditions that promote cell expansion but not cell differentiation.
16. The method of any one of claims 1-14 wherein the cells are cultured under conditions that promote cell differentiation.
17. The method of claim 16 wherein the cells are cultured in an environment comprising one or more of the following: growth factors, cytokines, extra-cellular matrix molecules, drugs, low molecular weight molecules or compounds, retinoic acid, DMSO .
18. The method of claim 16 or 17 wherein differentiated spheroid-forming cells of interest are selected and harvested.
19. The method of claim 18 wherein the cells of interest are selected on the basis of cell surface markers.
20. The method of claim 18 wherein the differentiated cells of interest are selected from the group consisting of: cardiac myocytes, vascular progenitor cells, neurons, hepatocytes, glial cells, kidney cells, beta cells and derivatives thereof.
21. A method for the selection of specific cell types from spheroid-forming cells comprising the steps of:
 - i. introducing a reporter gene expressing vector into at least one spheroid-forming cell whereby a cell-type specific promoter is combined with an reporter gene, such that the reporter gene is expressed under the control of the cell-type specific promoter;

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- ii. culturing the spheroid-forming cell(s)
- iii. differentiating spheroid-forming cell(s) in accordance with the method of claim 16
- iv. isolating and harvesting the specific cell-type based on the reporter gene expression

22. The method for the selection according to claim 21, wherein the reporter gene is an antibiotic resistance gene and the cell-type of interest is isolated by the addition of an appropriate antibiotic in step (iii) or/and (iv).

23. The method for the selection according to claim 22, wherein the antibiotic resistance gene is selected from but not limited to the group consisting of the Hygro mycin resistance gene (hph), the Zeocin resistance gene (Sh ble), the Puromycin resistance gene (pacA), and the Gentamycin of G418 resistance gene (aph).

24. The method for the selection according to claim 21, wherein the reporter gene is selected from but not limited to the group consisting of luciferase, green fluorescence protein, red fluorescence protein, and yellow fluorescence protein and the cells of interest are selected by fluorescent activated cell sorting (FACS).

25. The method for the selection according to claim 21, wherein the reporter gene is selected from but not limited to the group consisting of luciferase, green fluorescence protein, red fluorescence protein, and yellow fluorescence protein and the cells of interest are selected by fluorescent activated cell sorting (FACS).

26. The method for the selection according to claim 21, wherein the reporter gene is selected from but not limited to the group consisting of luciferase, green fluorescence protein, red fluorescence protein, yellow fluorescence protein, or a his-, myc-, or flag-tag ligated to a heterologous gene, or any heterologous gene which when expressed is inserted into the cell surface and the cells of interest are isolated from the cultured cells by affinity purification.

27. The method for the selection according to claim 21, wherein the cell type specific promoter is selected from but not limited to the group consisting of alpha-cardiac myosin heavy chain, MLC-2v, hANF, SOX2, HIP, PDX1,

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VE-Cadherin, human VE-Cadherin-2 and the selected cell type of interest is a cardiomyocyte, neural precursor, beta cell, endothelial cell.

28. The method of any one of claims 1-27 wherein the spheroid-forming cells are pluripotent cells.

5 29. The method of claim 28 wherein the pluripotent cells are selected from the group consisting of embryonic stem cells, embryonic germ layer cells, early primitive ectoderm-like cells, neural stem cells and adult pluripotent cells, adult mesenchymal stem cells, ductal setm cells, muscle derived stem cells, multiple adult progenitor cells.

10 30. The method of claim 29 wherein the pluripotent cells are embryonic stem cell and the spheroid is an embryoid body.

31. Embryonic stem cell derived cell culture obtained using the method of any one of claims 1-30.

15 32. The Spheroid-forming cell derived cell culture obtained using the method of anyone of claims 1-30.

33. A method to identify factors that effect cell proliferation, differentiation and/or spheroid formation, said method comprising culturing the cells as per the method of any one of claims 1-30 in the presence of the factor to be tested and then monitoring the effect of the factor on cell proliferation, differentiation and/or spheroid formation.

20 34. A method of generating cells derived from spheroid-forming cells in accordance with any one of claims 1-30 wherein the spheroid-forming cells are cultured under conditions that enable spheroid formation, said conditions comprising culturing said spheroid-forming cells in liquid suspension under non-aggregating conditions.

25 35. A culture bioreactor for industrial production of cells derived from spheroid-forming cells comprising:

- 30 c. culturing spheroid-forming cells in a spheroid-forming cell suitable media under conditions that promote spheroid formation; while
- d. Inhibiting spheroid aggregation.

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36. A method to prevent aggregation between cells comprising encapsulating a cell or group of cells and thus preventing aggregation of said cells with cells not within said capsule.

5 37. A bioreactor for generating cells from spheroid-forming cells comprising a means for controlling conditions suitable for spheroid formation, such conditions comprising a means for controlling cellular aggregation and means for maintaining said cells and generated cells in suspension.

38. The bioreactor of claim 37 wherein said means for controlling cellular aggregation is means for preventing cellular aggregation.

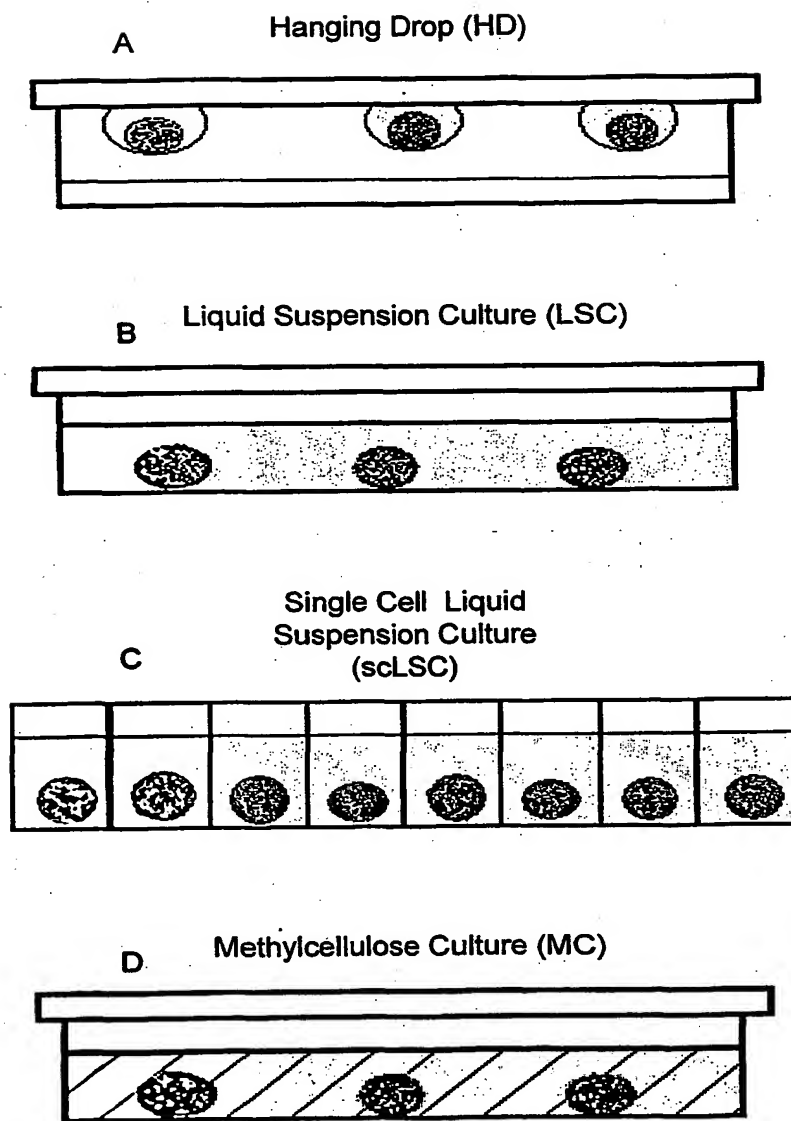
10 39. The bioreactor of claim 37 or 38 which is seeded with at least one spheroid-forming cell.

40. The bioreactor of claim 37, 38 or 39 wherein at least one spheroid-forming cell is encapsulated.

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FIGURE 1



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FIGURE 2

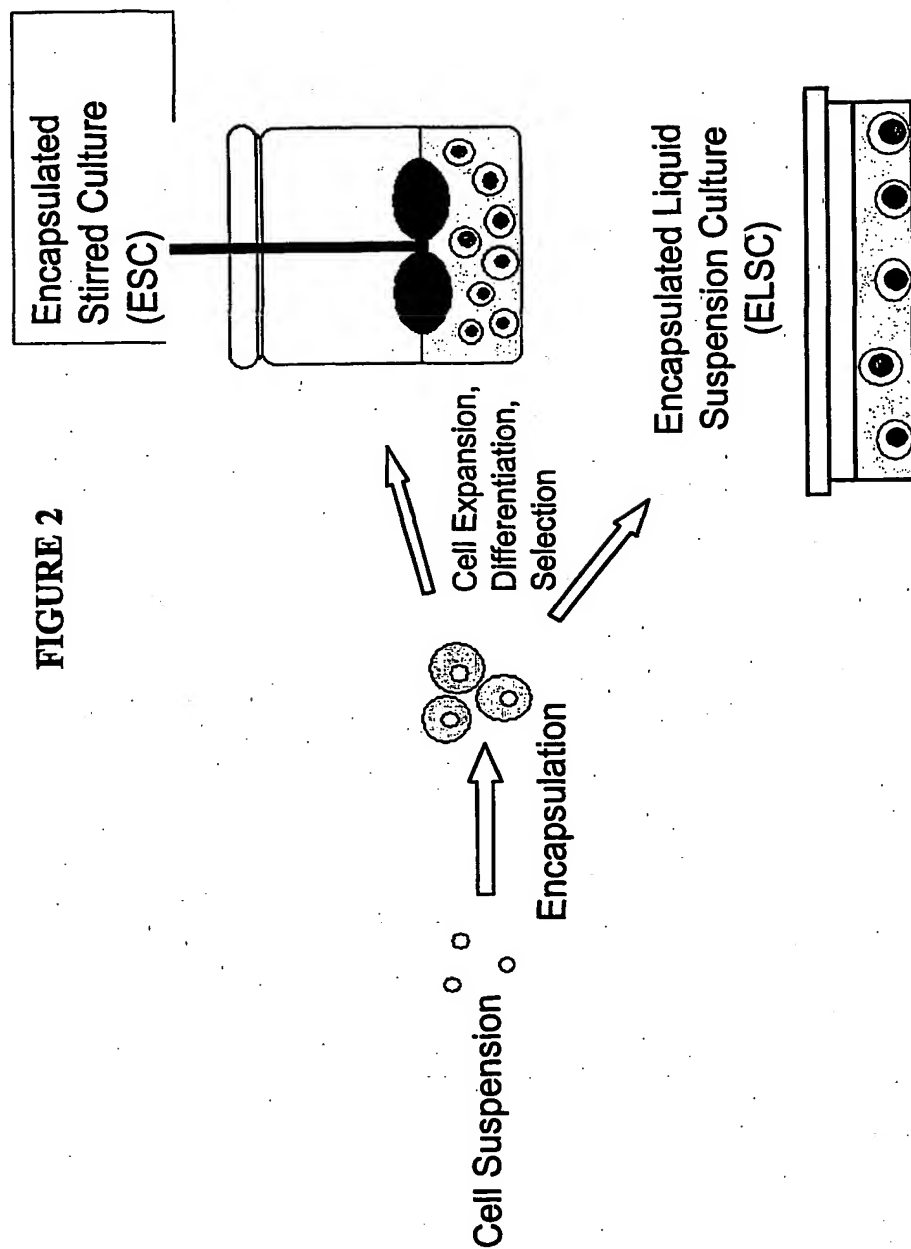
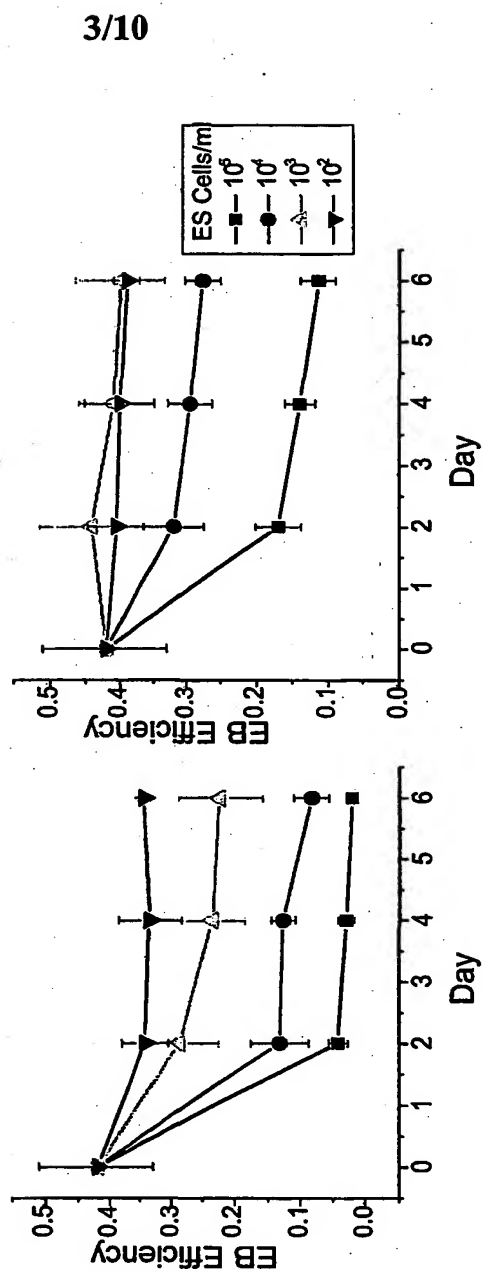


FIGURE 3



B

A

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FIGURE 4

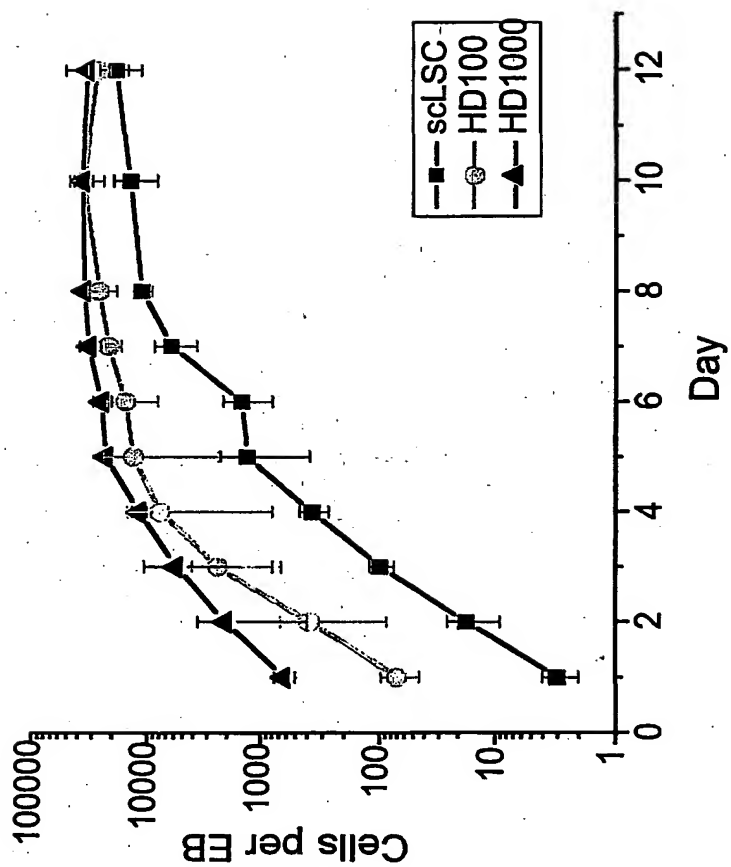
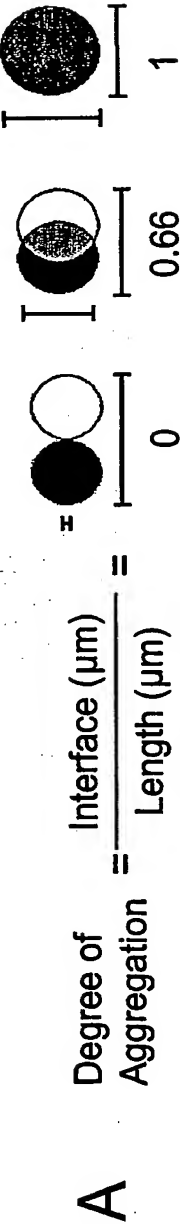
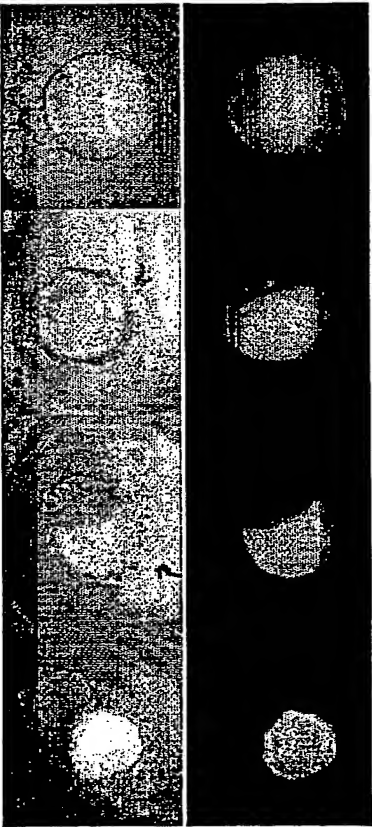


FIGURE 5



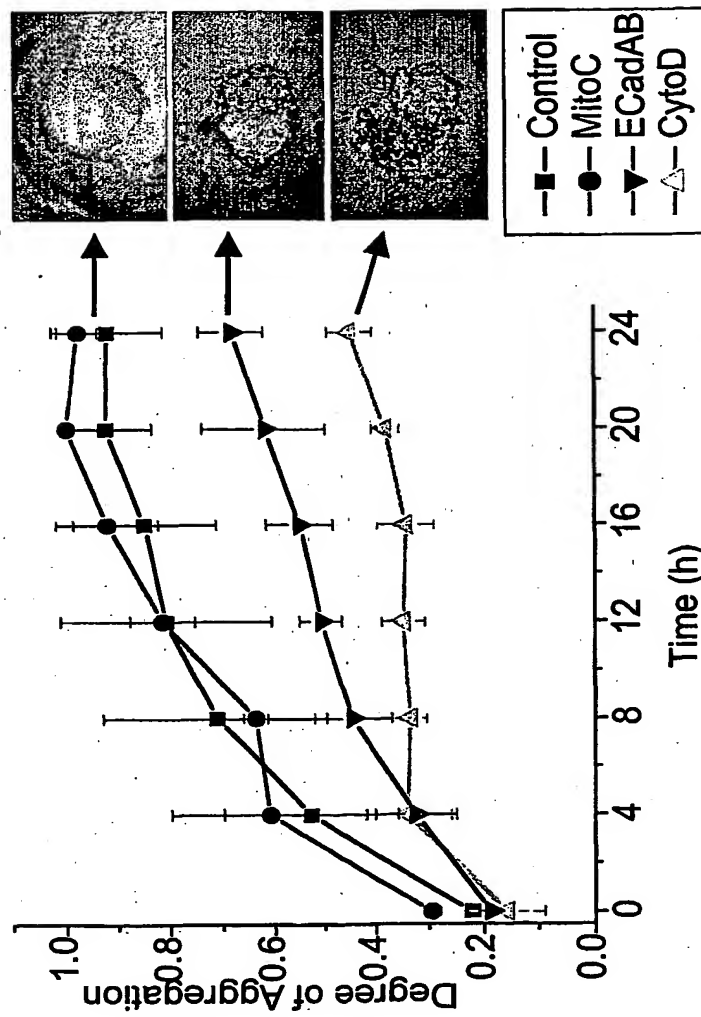
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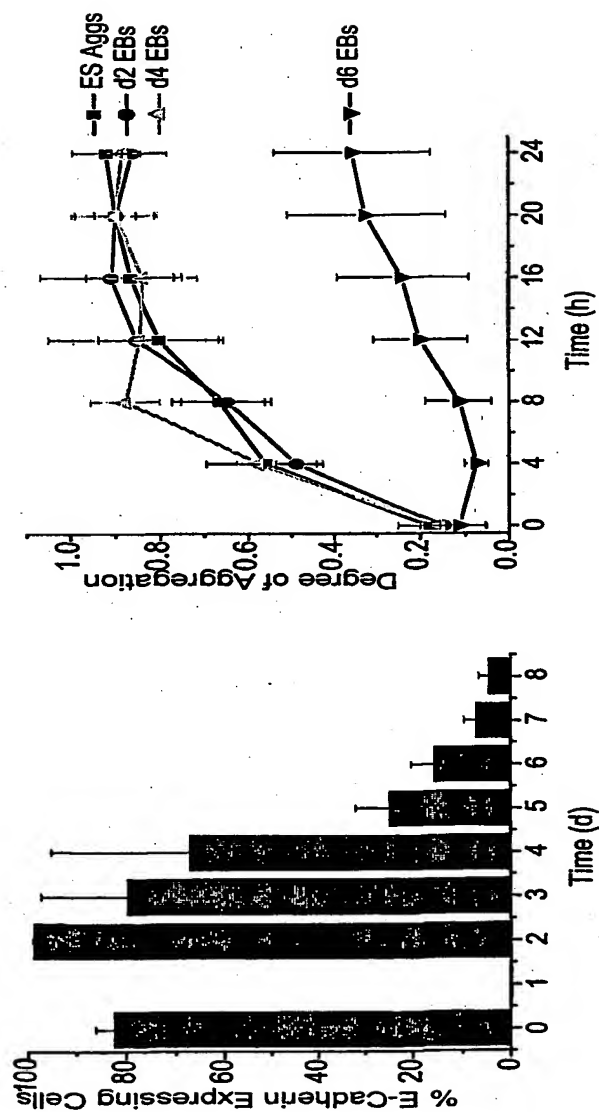
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FIGURE 6



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Figure 7

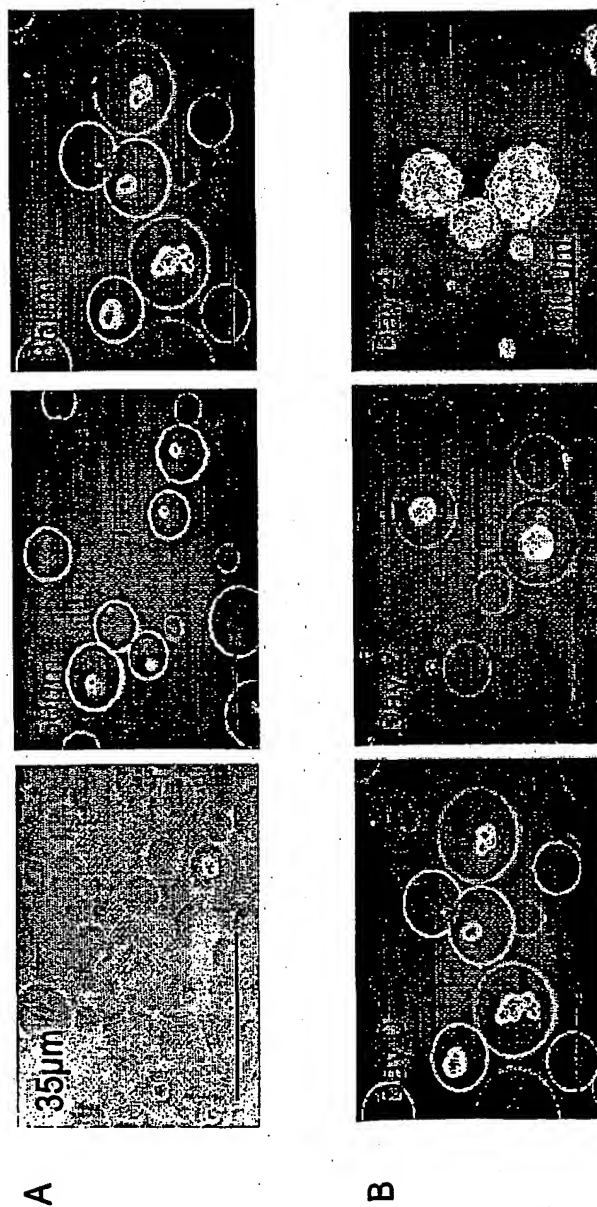


B

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FIGURE 8

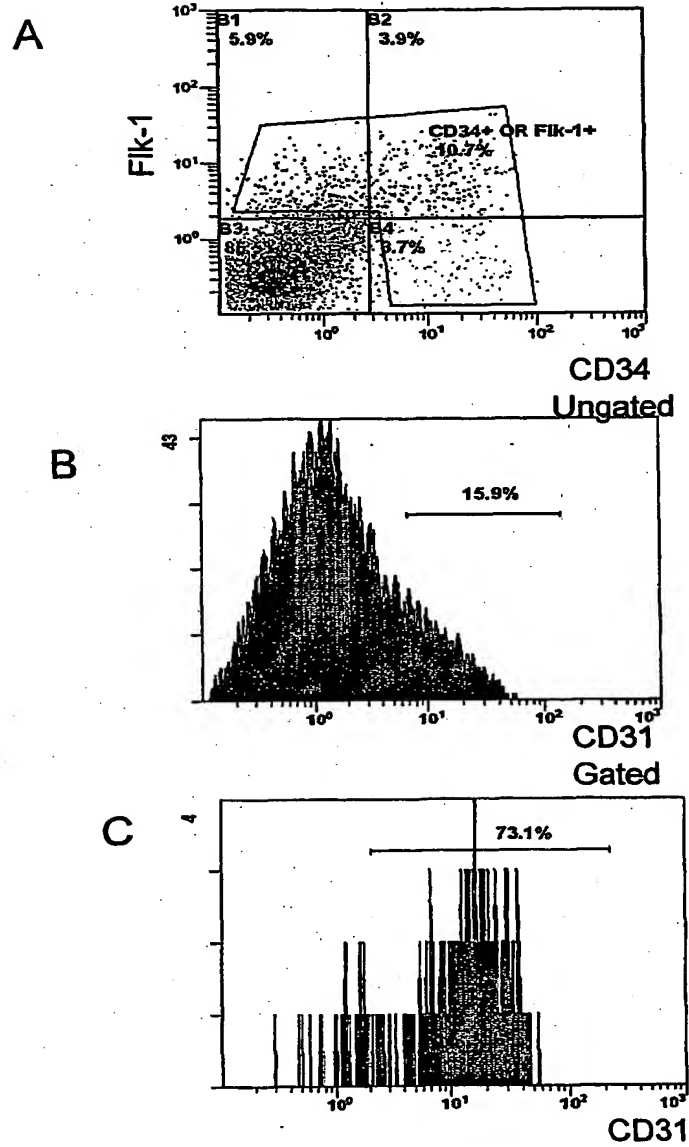


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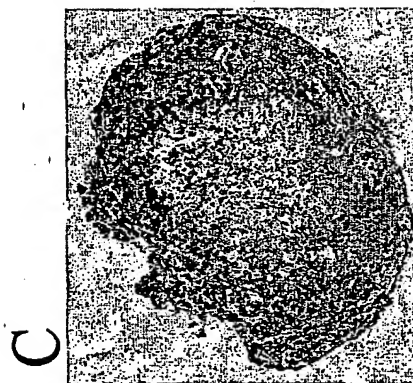
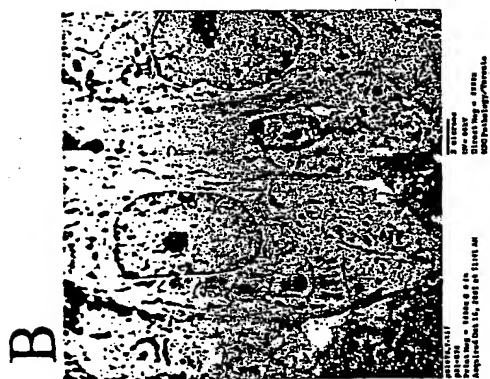
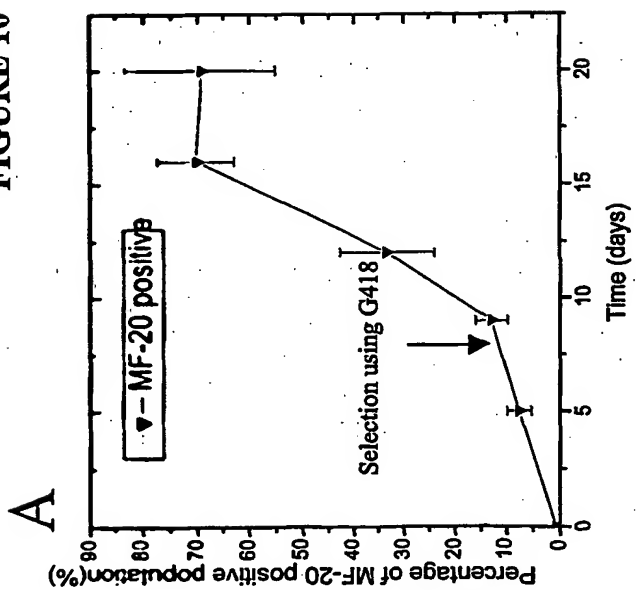
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FIGURE 9



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FIGURE 10



INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA02/01022

A. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

Name and mailing address of the ISA/

Authorized officer

Facsimile No.

Telephone No.

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 January 2003 (16.01.2003)

PCT

(10) International Publication Number
WO 03/004626 A2

(51) International Patent Classification⁷: C12N 5/06,
C12M 3/02, A01K 67/027

Stephen [CA/CA]; 58 Ambrose Road, North York, Ontario M2K 2W9 (CA).

(21) International Application Number: PCT/CA02/01022

(74) Agent: BERESKIN & PARR; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).

(22) International Filing Date: 4 July 2002 (04.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2,351,156 4 July 2001 (04.07.2001) CA
60/302,706 5 July 2001 (05.07.2001) US
10/189,642 3 July 2002 (03.07.2002) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(71) Applicant (*for all designated States except US*): CARDION AG [DE/DE]; IPR, Max-Planck-Str. 15a, 40699 Erkrath (DE).

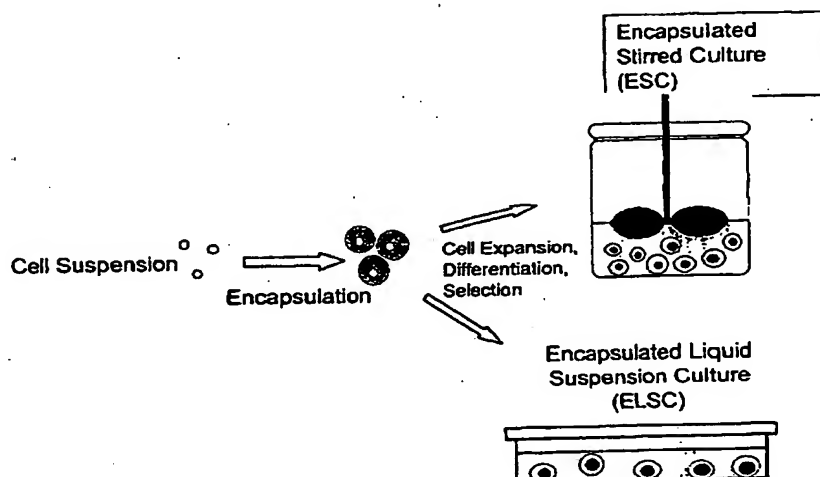
(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicants and

(72) Inventors: ZANDSTRA, Peter, W. [CA/CA]; 84 Alcina Avenue, Toronto, Ontario M6G 2E6 (CA). DANG,

[Continued on next page]

(54) Title: A BIOPROCESS FOR THE GENERATION OF CELLS DERIVED FROM SPHEROID-FORMING CELLS



(57) Abstract: The present inventors identified aggregation of embryonic stem cells and embryoid bodies (EBs) as the cause of the difficulty in generating large numbers of the embryonic stem cells (ES) cell-derived tissues. To counter this, the invention provides a novel bioprocess where aggregation of spheroid-forming cells, such as embryonic stem cells and spheroids, such as EBs is controlled, such as by encapsulation of within a matrix. As a result, EBs can be generated with high efficiency and cultured in high cell density, well-mixed systems. Well-mixed conditions facilitate measurement and control of the bulk media conditions and allow for the use of scalable bioreactor systems for clinical production of tissue. Therefore, the invention enables generation of ES cell-derived tissue on a clinical scale. The invention is also applicable to any spheroid-forming cells and other types of pluripotent cells.

WO 03/004626 A2



Published:

— *without international search report and to be republished upon receipt of that report*

(48) Date of publication of this corrected version:

6 March 2003

(15) Information about Correction:

see PCT Gazette No. 10/2003 of 6 March 2003, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

A BIOPROCESS FOR THE GENERATION OF CELLS DERIVED FROM SPHEROID-FORMING CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application hereby claims the benefit of priority from Canadian Patent Application No. 2,351,156 filed July 4, 2001 and United States Provisional Patent Application No. 60/302,706 filed July 5, 2001, both entitled, " A Bioprocess For The Generation Of Pluripotent Cell Derived Cells And Tissues". Both of these references are herein incorporated by reference.

10

FIELD OF THE INVENTION

 The invention relates to a process for generating cells and tissues, preferably cells and tissues derived from pluripotent cells or spheroid-forming cells. In one embodiment the pluripotent cells are embryonic stem cells.

15 Products and applications resulting from said process are also encompassed within the scope of this application.

BACKGROUND OF THE INVENTION

 Tissue engineering, transplantation therapy, gene therapy, and drug

20 discovery promise to revolutionize the medical paradigm and herald the coming of the "biotechnology era". However, development and implementation of these technologies depend upon cell-based approaches that require a plentiful supply of human cells: a supply that unfortunately does not currently exist. Therefore, identifying sources of human cells that can be

25 practically obtained in sufficient quantities is of paramount importance. Spheroid-forming cells, preferably pluripotent cells such as human embryonic stem cells (Thomson, Itskovitz-Eldor et al. 1998), neural stem cells (Clarke, Johansson, et al. 2000), multipotent adult progenitor cells (Schwartz, Reyes, et al. 2002), and others may be able to provide tissue in this manner.

30 The potential utility of pluripotent cells, in particular, the utility of embryonic stem (ES) cells, is derived from their demonstrated ability to differentiate into any other cell type in the body (Evans and Kaufman, 1981). Derived from the early embryo, ES cells can be grown and manipulated *in*

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vitro. ES cells are pluripotent cells that have the ability to differentiate into any cell type in the body (Evans and Kaufman 1981). They also have the ability to grow and multiply indefinitely while maintaining their pluripotentiality. Theoretically, these traits make ES cells an unlimited source of any cell type and therefore a very attractive tissue source for other biotechnological applications (Zandstra and Nagy 2001).

Generation of various differentiated cell types (cardiac myocytes, hematopoietic cells, neurons, hepatocytes, and others) from murine ES cells (e.g., Wang, Clark et al. 1992; Rohwedel, Maltsev et al. 1994; Bain, Kitchens et al. 1995; Palacios, Golunski et al. 1995; Choi, Kennedy et al. 1998; Fleischmann, Bloch et al. 1998; Tropepe, Sibia et al. 1999) as well as from human ES cells (e.g., Jones and Thomson 2000; Thomson et al. 1998; Mummery et al 2002; Levenberg et al. 2002; Reubinoff et al. 2001; Assady et al 2001) has been extensively reported.

In general, production of these various cell types from ES cells require a complex multi-step process, starting with the expansion of ES cells to adequate numbers. Next, ES cells would be differentiated to the desired cell type(s). Finally, the desired cells would be selected and purified from the remaining cells before they could be utilized for various applications.

In vitro, differentiating ES cells follow a reproducible temporal pattern of development that in many ways recapitulates early embryogenesis (Keller 1995). As ES cells and their derivatives proliferate and differentiate, they typically form spherical tissue-like structures (spheroids) called Embryoid Bodies (EBs). Other pluripotent stem cells such as neural stem cells have also been described to form spheroids. In the case of neural stem cells these spheroids are termed neurospheres (Kalyani, Hobson, and Rao, 1997). In the case of ES cells, over time, EBs increase in cell number and complexity as cells from the three embryonic germ layers are formed (Keller 1995). The ability to control and manipulate the formation of spheroids from pluripotent cells and other types of cells that are derived directly, or indirectly from human tissue, as well as the interactions between the spheroids and between the spheroid-forming cells (i.e., prevent or inhibit the aggregation of spheroids) would allow for the control of interactions between these cells during

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proliferation and differentiation, and thus be an important development for the design of technologies for the *in vitro* differentiation of pluripotent cells. For example, the generation of "chimeric spheroids" or "chimeric EBs" defined as multicellular, spherical structure comprising a mixture of differentiated cells and pluripotent cells (Perkins, 1998), has been shown to be an effective approach to influence the development of the cell types in from pluripotent cells (Clarke, Johansson et al. 2000).

Current ES cell differentiation systems include cultures initiated with an ES cell suspension in liquid media (LSC), or methylcellulose (semi-solid) media (MC), or attached to a surface in liquid media, or with multi-ES cell aggregates formed in "hanging drop" cultures (HD) where ES cells are aggregated in hanging drops for 2 days before transfer to liquid culture (see Figure 1). These systems are adequate for small-scale laboratory purposes but are not amenable to clinical production because of deficiency in three important areas: a) the ability to measure and control the extracellular environment, b) scalability, and c) cell density.

These systems are static and batch-style cultures that result in formation of spatial and temporal gradients in nutrients and metabolic products. These gradients make measurement and process control difficult because these processes depend on sampling measurements that need to reflect the conditions throughout the culture. The inability to control the cell culture environment will affect product purity and reproducibility, as well as the cell types that can be readily generated [e.g., for oxygen (Maltepe, Schmidt, et al. 1997), glucose (Soira, 2001) and many other biological and physicochemical factors (Zandstra and Nagy, 2001)]. In the case of methylcellulose culture, the semi-solid media also hinders measurement and manipulation. Regulatory approval for the use of any biotechnology product demands consistency in the method of production and in the final product itself (<http://www.fda.gov/cdrh/tisseng/te6.html>). This is not possible when measurement and control are impeded or absent.

Second, clinical or industrial scale production of cells from the current culture systems is not practical. The current systems are typically carried out in petri dishes where cells grow in only a thin layer of media. These systems

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are essentially two-dimensional with respect to the EBs; therefore, unrealistically large surface areas would be required for industrial scale production. It can be estimated that several billion cells would be need for applications such as cardiac cell therapy (Zandstra and Nagy, 2001); this
5 scale of cell production is highly impractical, if not impossible using current technologies.

Third, current liquid suspension cultures (LSC) require EBs to be cultured at relatively low density. Higher cell densities can be achieved in methylcellulose cultures, where semi-solid media reduces the likelihood of cell
10 aggregation, however media supplementation of methylcellulose cultures is troublesome and increased cell density would demand more frequent supplementation.

Therefore, there is a need to develop methods for efficient growth and differentiation of spheroid-forming cells, preferably pluripotent cells, such as
15 ES cells.

SUMMARY OF THE INVENTION

Because of the limitations reviewed above, one ideal differentiation culture system for clinical scale production of EBs or other spheroids would be
20 the stirred tank reactor or other scalable, well-mixed and controlled bioreactor systems such as a fluidized bed reactor. While the standard methods are limited to two-dimensional growth because of static conditions, a controlled bioreactor could suspend the cells evenly throughout the volume (in three-dimensions). This configuration of system can be easily scaled in size to
25 accommodate the need for increased production of cells. In addition, stirring ensures homogenous media conditions throughout the reactor that would facilitate measurement and control of the extracellular environment.

Despite the importance of stirred or other well-mixed bioreactors, to date there have been no reports of successful use of these systems for ES
30 cell differentiation culture. ES cells added directly to stirred culture quickly aggregate into large cell clumps within 24 hours and cell growth and differentiation within these clumps are severely impaired (Wartenberg et al. (1998)). However, it was not known whether aggregation was the cause or

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result of a different problem. Differentiation of ES cells in stirred culture is therefore a non-trivial task; however the ability to do so would greatly facilitate clinical and industrial scale production of cells for therapeutic purposes. ES cells cultured in stirred bioreactors fail to generate EBs in an efficient manner.

5 This patent application is based upon a novel technology that overcomes one or more of the limitations of the prior art: *a)* the ability to measure and control the extracellular environment, *b)* scalability, and *c)* cell density and allows for the scalable generation of cells and/or tissue derived from spheroid-forming, preferably pluripotent, preferably ES cells.

10 The present inventors have determined that ES cells cultured in stirred bioreactors fail to generate EBs in an efficient manner because the cells aggregate forming large cell clumps that results in poor cell proliferation and incomplete cell differentiation and that controlling aggregation can overcome this problem. As such, in one aspect, the present invention provides a process
15 to control cellular and spheroid aggregation of spheroid-forming cells, such as ES cells or neuronal stem cells, to enable the use of a stirred tank reactor or other scalable, well-mixed and controlled bioreactor systems such as a fluidized bed reactor or other liquid suspension system. The invention can also have benefits in other systems, especially liquid systems such as LSC.

20 In one embodiment, the invention provides a method of culturing spheroid-forming cells, preferably pluripotent cells, preferably ES cells, under controlled cell aggregation conditions that enable spheroid formation. In another embodiment the method is done in a bioreactor system by controlling cellular aggregation. In one embodiment the cells are differentiating cells. In
25 yet another embodiment, the invention provides a method of generating or obtaining cells derived from spheroid-forming cells cultured under controlled aggregation conditions. Although a preferred embodiment of the invention is with ES cells, the invention is not limited to ES cell derived cells and tissues. It is equally applicable to any spheroid-forming cell type, preferably pluripotent
30 cell type that can differentiate as a cell cluster or aggregate, or form spheroid bodies, such as adult pluripotent cells (Schwartz, Reyes, et al. 2002; Clarke, Johansson et al. 2000) embryonic germ cells (EG cells) (reviewed in Thomson and Odorico, 2000), early primitive ectoderm-like cells (EPL) (Pelton

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and Sharma, 2002), and neuronal stem cells. In one embodiment, the invention can also apply to other cell types where cell aggregation or control of the cell microenvironment is indicated. As such the embodiments of the invention as outlined below in relation to embryonic stem cells also apply to other pluripotent cells where controlling aggregation of the cells at various points of expansion and or differentiation can enhance the efficiency of said expansion, spheroid formation or differentiation and are intended to be encompassed within the scope of the present invention.

In one embodiment, the invention provides a method of generating cells derived from spheroid-forming or pluripotent cells comprising culturing the cells, for example pluripotent cells, such as ES cells, EG cells, EPL cells, and adult pluripotent cells (Schwartz, Reyes, et al. 2002), while controlling cellular and spheroid aggregation. In a preferred embodiment, the method is conducted under conditions that permit cell differentiation and proliferation. In another embodiment, the cells are encapsulated to prevent aggregation with neighboring cells, spheroids or cells contained in separate capsules. In another embodiment the aggregation between specific cell types is controlled (to enable aggregation necessary for spheroid formation, where applicable) and the aggregation between spheroids of these cells is prevented. In yet another embodiment the kinetics of aggregation is controlled.

In one embodiment, the invention provides a method of generating cells from spheroid-forming cells, preferably embryonic stem cells, comprising culturing the spheroid-forming, preferably embryonic stem cells under conditions that enable spheroid or embryoid body formation and cell differentiation while controlling cell aggregation.

In a preferred embodiment, the spheroid-forming cells, such as embryonic stem cells are encapsulated to control cell aggregation such that each capsule will transiently contain and give rise to one spheroid or embryoid body. In another embodiment, each capsule contains a predetermined or desired number of spheroid-forming or ES cells that are permitted to aggregate and together give rise to a single spheroid or EB. Preferably, the undifferentiated spheroid-forming cells, such as embryonic stem cells are first encapsulated to prevent aggregation between the cells contained within

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separate capsules. The encapsulated cells are then cultured under conditions that enable cell proliferation and optionally differentiation, leading to spheroid or embryoid body formation. In a more preferred embodiment, emulsification of cells with agar in inert silicon oil is used to encapsulate the cells. In a most preferred embodiment, this emulsification results in the generation of agarose microcapsules containing cells. In yet another embodiment the process of encapsulation allows for the control of spheroid-forming cell aggregation by interfering with cell surface receptor binding. In one embodiment, the process of encapsulation allows for the control of spheroid-forming or ES cell aggregation by interfering with E-cadherin mediated cell aggregation.

In another embodiment, the invention provides a method wherein the embryonic stem cells are cultured under conditions wherein embryoid bodies and/or differentiated embryonic stem cells can be formed. For example, in some ES cell lines multiple ES cells may be preferred for EB formation. Following these permissive steps, cells are transferred to conditions that prevent aggregation of embryoid bodies. In one embodiment, the method further comprises a step wherein the differentiated embryonic stem cells and/or tissues of interest are selected and harvested. In a preferred embodiment, the cells and/or tissues of interest are cardiomyocytes or cardiac tissue or hematopoietic cells or tissue or endothelial cells or tissues.

In another embodiment, the invention provides an embryonic stem cell derived cell culture obtained using the method of the invention. In a further embodiment, the invention provides embryonic stem cell derived cells and tissues obtained using the method of the invention.

In yet another embodiment the invention provides a method to identify factors, e.g. any variant, condition, substance, that affect embryonic stem cell differentiation and/or embryoid body formation, said method comprising culturing the embryonic stem cells while preventing embryoid body and embryonic stem cell aggregation, in the presence of the factor to be tested and then detecting the effect of the variant on embryonic stem cell differentiation and embryoid body formation. In a further embodiment, of said method, the effect on embryonic stem cell differentiation and embryoid body formation is compared to a control culture, preferably a negative control

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wherein embryonic stem cells are cultured under the same conditions except in the absence of the factor to be tested. The invention also provides a method of identifying factors that affect cell proliferation or differentiation in any cell culture by encapsulating said cells. The invention also encompassed
5 a method of preventing cell aggregation in any cell type by encapsulation.

Applications and uses of the method and initiating and resulting cells, tissues, cultures and bioreactors or culture systems are also encompassed within the scope of the present invention.

Other features and advantages of the present invention will become
10 apparent from the following detailed description. For instance where reference is made to embryoid bodies or embryonic stem cells, the invention can also be applied to spheroid-forming cells and spheroids. It should be understood, however, that the detailed description and the specific examples while
15 indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

20 The invention will now be described in relation to the non-limiting drawings in which:

Figure 1 is a schematic drawing of the current technologies used to culture pluripotent stem cells. (A) Hanging drop (HD) cultures typically consist of a
25 defined number of cells allowed to aggregate in small fluid volumes that hang from the tops of tissue culture dishes. Liquid suspension cultures (LSC) (B) are typically low-density suspension cultures of individual cells or aggregates of cells. When individual ES cells are put into the bottoms of 96 well plates to measure EB developments these cultures are termed single cell liquid
30 suspension cultures (scLSC) (C). Methycellulose (MC) (or similar solid or semi solid cultures) are cultures where cells are enveloped in semi- or semi-solid media that prevents aggregation, for example by preventing the collision of cells or cell aggregates due to its high viscosity (D).

Figure 2 is a schematic diagram of one embodiment of the method to control spheroid-forming cell and/or pluripotent cell and spheroid aggregation. Test cell populations (either individual cells or controlled aggregates of cells) are encapsulated, put inside a controlled stirred suspension bioreactor (Encapsulated Stirred Culture (ESC)), or in any type of encapsulated liquid suspension culture (ELSC) where, because of the encapsulation technology, they are prevented from aggregating, and allowed to proliferate and differentiate into the desired cell type.

10

Figure 3 is a graph illustrating the affect of initial ES cell density (i.e., number of ES cells per unit volume) on EB efficiency (ratio of number of cell aggregates to number of input ES cells) over time for A: liquid suspension culture (LSC) and B: methylcellulose (MC) culture. Cultures were initiated with ES cell densities: 10^2 , 10^3 , 10^4 , 10^5 cells/ml.

15

Figure 4 is a linear graph illustrating cells per EB (y-axis) over time (x-axis). EBs were initiated with: 1 (scLSC), 100 (100 cells per drop in the hanging drop cultures, HD100), and 1000 (1000 cells per drop in the hanging drop cultures, HD1000) ES cells/EB. Despite initial differences in cells/EB, all EBs grew to approximately the same size by day 12.

20

Figure 5 (A) illustrates the method of quantifying degree of aggregation (DOA) between two EBs, defined as the ratio of the diameter of the interface to the overall length of the two EB system. DOA is calculated by dividing the interface diameter by the overall length of the two EB system, yielding a scale between 0 (no aggregation) and 1 (fully aggregated). (B) A representative photomicrograph series of two EBs aggregating over time is shown with corresponding DOA measurements at each time interval. Two types of ES cell derived EBs were used in this experiment, one that was not fluorescent and one that was constitutively fluorescent (light EB in the photomicrograph)).

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Figure 6 is a linear graph illustrating the effects of the chemical additives cytochalasin D (CytoD), mitomycin C (MitoC), E-cadherin blocking peptide (ECadAB), and a control, on the aggregation kinetics, measure using the DOA, of EBs. A representative photo taken at 24 hours of culture is shown for each treatment group.

Figure 7 panel A is a bar graph illustrating percent E-cadherin expressing cells (y-axis) versus time (x-axis). Figure 7 panel B is a linear graph illustrating aggregation kinetics of EBs differentiated for 0, 2, 4, and 6 days.

Figure 8 panel A are photographs of ES cells encapsulated inside agarose microcapsules designed to have specific diameters. Shown are capsules of mean diameter 35, 50, and 85 μm . The scale is indicated by the straight lines at the bottom of each photograph which each represent 100 μm . In some capsules single ES cells were encapsulated ((35 and 50 μm capsules), while in other instances, a plurality of ES cells were encapsulated (85 μm capsules). Figure 8 panel B is a photograph series of differentiating ES cells forming EBs inside the agarose microcapsules. Day 0 shows encapsulated ES cells, day 2 shows day 2 EBs, day 4 shows EBs emerging, as desired, from the agarose microcapsules.

Figure 9 is a series of flow cytometry plots showing phenotypic detection of endothelial cells generated from encapsulated ES cells grown in stirred suspension bioreactors using flow cytometric based cell surface expression of various endothelial cell markers. Figure 9 panel A shows cells expressing Flk-1 (y-axis) and CD34 (x-axis). Cells expressing either Flk-1 or CD34 were gated and the percent of cells expressing these markers is indicated. Figure 9 panel B shows CD31 expression by ungated cells. Figure 9 panel C shows CD31 expression by gated cells. CD34 and/or Flk-1 expression together with CD31 expression identifies endothelial cells.

Figure 10 Panel A shows kinetic changes of the percentage of the MF-20 positive cells during the course of differentiation and selection with G418 of

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transgene – containing ES cells cultured in stirred suspension bioreactors. Figure 10 panel B shows morphologic and structural analysis of bioreactor – grown ES cell-derived cardiomyocytes. The cells exhibit a mature sarcomeric organization and Z-banding (arrow). Figure 10 panel C shows a cross section of a “cardiac body” (a spheroid of ES cell derived cardiac cells) generated in bioreactors.

10 DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

“Adult stem cell” as used herein means an undifferentiated cell found in a tissue in an adult organism that can renew itself and can (with certain limitations) differentiate to yield specialized cell types of the tissue from which it originated or to yield specialized cell types in other tissues and organs.

“Pluripotent cell” as used herein refers to a cell that can self renew and differentiate as a cell cluster or aggregate into one or more types of cells and/or tissue. Examples of such cells include but are not limited to embryonic stem cells, embryonic germ cells, early primitive ectoderm-like cells and derivatives, multiple adult progenitor cells, adult or embryonic neural stem cells (NSC) (Toma and Akhavan, 2001; Hitoshi, Tropepe, Sibilia, et al. 1999) adult mesenchymal stem cells (MSC), mesenchymal adult pluripotent stem cells, sometimes referred to as MAPC (Schwartz, Reyes, et al. 2002), ductal stem cells (Suzuk, Zheng et al. 2002), muscle derived stem cells (McKinney-Freeman, Jackson et al. 2002).

“Embryonic stem cell” as used herein refers to pluripotent cells derived from the inner cell mass of a blastocyst, or derived by other methodologies (Jones and Thomson (2000)) and shown to be able to contribute to development of multiple tissues.

“Embryonic stem cell derived cells and tissues” as used herein refers to any cells or tissues that are derived from embryonic stem cells. The term “pluripotent cell derived cells and tissues” as used herein refers to any cells or tissues that are derived from pluripotent cells or tissues. The term “spheroid-

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forming cell derived cells and tissues" as used herein refers to any cells or tissues that are derived from spheroid-forming cells or tissues.

"Spheroid-forming cell" as used herein means cells that form multi-cellular aggregates consisting of more than a single cell when cultured in suspension

5 "Spheroid" as used herein means a cellular structure consisting of more than a single cell that has initially developed from a single or from multiple cells.

"Embryoid Body" as used herein means a "Spheroid" which is derived from embryonic stem cells.

10 "Clonal Spheroid" as used herein means a cellular structure consisting of more than a single cell but which has been developed from a single cell.

"Stem cell" as used herein means an "adult stem cell" or a "pluripotent cell".

15 "Chimeric spheroid" is a "spheroid" made up of at least two distinct cell types. For example, in one embodiment it has been shown that the mixing ES cells with adult pluripotent cells can broaden the differentiation capacity of the adult pluripotent cells, for instance a chimeric spheroid can comprise two different pluripotent cell types, one an adult pluripotent cell (e.g. neuronal pluripotent cell) and the other an ES cell. In one embodiment the ES cell can induce the other cell type to differentiate into other cell types such as cardiac

20 cells.

DESCRIPTION

The present invention is directed to a new method for generating spheroid-forming cell and/or pluripotent cell derived cells and tissues, such as

25 for example cardiomyocytes, hematopoietic cells, endothelial cells, insulin producing beta cells, neuronal cells, glial cells, kidney cells, hepatocytes, vascular progenitor cells or derivatives (such as hematopoietic stem cells or endothelial cells), to name a few.

30 The present inventors have shown that as cell density increases, cell aggregation occurs more readily, resulting in lower cell expansion and impaired cell differentiation (also see Dang, et al. 2002). The present inventors have also hereby shown that cellular and spheroid aggregation is indeed the cause of the inability to culture spheroid-forming cells in a high

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density culture, such as in a liquid suspension culture (e.g. a stirred liquid bioreactor system). As such the inventors have shown that controlling cellular and/or spheroid aggregation can increase spheroid-forming efficiency.

Thus in one embodiment, the invention provides a method of
5 generating cells and/or tissues derived from spheroid-forming cells, preferably pluripotent cells comprising culturing spheroid-forming cells under controlled cell aggregation conditions. In another embodiment, such conditions enable spheroid formation in liquid suspension.

In a preferred embodiment, the invention provides a method for
10 efficient formation of spheroids or EBs and the culture of spheroid or EBs in suspension at higher cell densities. This is done by controlling cell aggregation. Thus, in another embodiment of the method of the invention, the spheroid-forming cells can be cultured under high density conditions. Although the invention will work at low cell density (even seeding the initial
15 culture with 1 cell), it can also work with an initial cell density of about 10^2 to about 10^6 cells/ml, preferably about 10^3 to about 10^6 cells/ml, more preferably from about 10^4 to about 10^6 cells/ml. The invention is not intended to be limited by such cell density values and are provided as a suitable example. These cells can then be cultured under controlled aggregation conditions to
20 yield to form spheroids. As one can start with a culture with more cells than the prior art, the resulting number of spheroids formed will be greater than in cultures commenced at a lower cell density. In one embodiment, each spheroid may be cultured to about 30,000 to 35,000 cells per spheroid, preferably about 30,000 to 46,000 cells per spheroid. It should be noted that
25 this is but one embodiment, and the invention is not limited to such numbers. A person skilled in the art could control conditions (such as time, temperature, media conditions, other environmental factors) to get lower or potentially higher numbers. Thus the present method permits generation of cells at an industrial scale.

30 In one embodiment, the invention provides a scalable method for generating spheroid-forming cell or pluripotent cell derived cells and tissues, such as spheroid or ES derived cells and tissues for various uses, such as for transplantation.

In another embodiment, the invention provides a method of culturing spheroid-forming cells, such as pluripotent cells in a bioreactor system where the culture conditions can be measured and controlled. In yet another embodiment, the invention provides a scalable and controllable culture of spheroid-forming cells, such as pluripotent cells by allowing them to be cultured in liquid suspension cultures, such as stirred bioreactors, such as stirred liquid bioreactors. This is done by controlling cell aggregation. This improves the efficiency of generation of differentiated cells.

In one embodiment, the bioreactor or culture system used in the invention is one that keeps the cells and/or spheroids in liquid suspension. In another embodiment, this culture system keeps the cells and/or spheroids in liquid suspension by stirring, but other methods or means can be used, such as by agitation of the system, use of various media or other environmental conditions.

The term "controlling cell aggregation" refers to the process of effectively modulating the extent and kinetics of cell aggregation such that an endpoint, not effectively attainable without such control, is achieved. As used herein in relation to cell and/or spheroid or EB aggregation, "controlling cell aggregation" means that cell aggregation can be permitted or prevented as desired. For example, in one aspect of the invention, aggregation of spheroid-forming cells, such as ES cells, sufficient to induce spheroid or EB formation is permitted, and aggregation between cells within a spheroid or EB is permitted. However aggregation between spheroid-forming cells or ES cells beyond that determined to induce spheroid or EB formation is prevented. Aggregation between separate EBs is also prevented.

Further, benefits of the invention (i.e. increasing efficiency of generating cells from spheroid-forming, pluripotent or ES cells) can be obtained with any degree of prevention of cellular and/or spheroid aggregation. Controlling aggregation may, in some application consist of preventing aggregation where the term "preventing" includes any inhibition of (i.e. that may prevent some, but not necessarily all) of spheroid-forming cell or pluripotent cell and/or spheroid or EB aggregation in a particular culture system. It would be appreciated by those skilled in the art upon reading the

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description herein that any prevention of spheroid, EB and/or spheroid-forming cell or pluripotent cell aggregation would improve cell yields in the culture system and/or cellular differentiation.

5 In one embodiment, "preventing, spheroid and/or ES cell and/or spheroid or EB aggregation" means that after spheroid-forming cells or ES cells are cultured to form spheroids or embryoid bodies, there may be cellular aggregation between cells within a spheroid or embryoid body but not between cells of different spheroids or embryoid bodies or between spheroid or embryoid body themselves.

10 In another aspect, the invention provides a method of generating cells derived from spheroid-forming cells wherein the spheroid-forming cells are cultured under conditions that enable spheroid formation, said conditions comprising culturing said spheroid-forming cells in liquid suspension under non-aggregating conditions.

15 Thus in one embodiment, the invention provides a method of culturing spheroid-forming cells or ES cells comprising culturing the cells under conditions that promote spheroid or EB formation, including controlled aggregation of spheroid-forming or ES cells, then preventing cell and spheroid or EB aggregation.

20 In another embodiment, the invention provides a method of controlling the production of spheroids from spheroid-forming cells by controlling the aggregation of such cells. In one embodiment, the spheroid-forming cells are pluripotent cells such as neural stem cells that form neurospheres, or are embryonic stem cell which form EBs.

25 In a preferred embodiment the method of the invention does not adversely affect spheroid or EB formation, cell growth, or cell differentiation. Thus in another preferred embodiment, the method does not adversely effect the delivery of desired nutrients, oxygen, or cytokines. In yet another embodiment, the method can further comprise culturing the spehroid-forming or ES cells under conditions that promote differentiation of the cells and
30 spheroid or EB formation. For instance, ES cells can be cultured in a suitable base media (such as ES cell media) without LIF. In another embodiment, the cells can be cultured under conditions that do not promote cell differentiation

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or further differentiation. For instance ES cells can be cultured in a suitable base media (Such as ES cell media) with LIF. Thus the method of the invention, by adjusting culture conditions can generate differentiated cells or be used to expand the existing cell culture initially or at any point of the method. For instance, in one embodiment culture conditions can change be changed during the method to control differentiation conditions. Examples of culture conditions are described further below.

10

Examples of conditions that can be used in the present invention for culturing spheroid-forming cells, such as ES cells to control spheroid or EB and/or cell aggregation include but are not limited to:

- 15 (a) Methods for preventing physical association between cell aggregation molecules (i.e. encapsulation, such as described herein or Sefton et al. 1997).
- (b) Methods that use reduced inorganic salt concentration as described in Boraston et al. 1992 and Ko et al. 2001.
- (c) Methods that block surface aggregation molecules with specific peptides or other molecules, such as described in Burdsal et al. 1993.
- 20 (d) Using genetically modified spheroid-forming or ES cells such as E-cadherin-null ES cells such as described in Larue et al. 1996 or methods that inhibit E-cadherin expression or antagonize or inhibit E-cadherin activity.
- 25 (e) Addition of agents that prevent cell aggregation such as dextran sulfate or other sulfated polyanions as described in Dee et al. 1997
- (f) Methods that prevent cell aggregation by physically separating one or more spheroid-forming cells to enable spheroid formation in distinct compartments such as described in the single cell liquid suspension culture (scLSC) method in this patent without limitation to a single cell.
- 30

In one embodiment of the invention, the above methods can be used alone or in combination, ie, more than one method can be used to prevent

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spheroid, EB and/or spheroid-forming or ES cell and/or non-clonal spheroid-forming or non-clonal ES cell aggregation.

The selected spheroid-forming or ES cell culturing strategy preferably does not affect the development and differentiation of pluripotent cells, preferably ES cells, or EBs in any undesired way.

In a preferred method of the invention, the spheroid-forming or ES cells are encapsulated. In one embodiment, the average number of initial cells encapsulated can be predetermined or conditions can be adjusted to get a desired number of cells per capsule (for instance the capsule matrix, cell density, media or other environmental or capsule forming conditions). For instance, in one embodiment, the number of cells per capsule is 1-10, 5-10, 1-5, 5, or 1, but the invention is not limited by such examples. In one embodiment, the spheroid-forming or ES cell or cells can be encapsulated using methods such as described in Weaver et al. 1988 or Turcanu et al. 2001 and then cultured under conditions that promote spheroid or EB formation and cellular differentiation, such as described in Keller et al, 1995 or O'Shea et al. 1999. In another embodiment, the capsule comprises cells of at least two different cell types, such as an ES cell and an adult pluripotent cell to produce a chimeric spheroid.

A variety of methods can be used to encapsulate cells. Although, the Examples described herein use the Gel Microdrop Technique (GMD), patented and owned by One Cell Systems, Boston Massachusetts. The GMD technique was designed to encapsulate cells in agarose gel for the purpose of isolating individual cells with specific protein secretion profiles; however, the present invention adapted the process for encapsulation of spheroid-forming or ES cells and controlling cell and spheroid or EB aggregation. It is important to note that the microencapsulation of spheroid-forming cells such as ES cells for the purposes of the present invention does not depend upon the GMD technique as any method of encapsulation could be used.

In one embodiment the size of the capsule can be controlled. In one aspect, this can determine the time at which a spheroid or EB emerges from the capsule. Preferably the size of the capsule is such that maintains the spheroid-forming cells and/or spheroids in the capsule until aggregation cell

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surface markers or other aggregating factors (molecules, proteins, etc..) are no longer expressed. For instance, under certain conditions, as noted in the present examples, for ES cells, EBs that emerge after about 4 days no longer express E-cadherin and thus do not have a tendency to aggregate. In one approach, varying the rate of emulsification during the encapsulation process can control the size of the microcapsule. The different capsule sizes produced under each of the conditions tested are shown in Table 3. Other factors that may control the size of the microcapsules include the addition of surfactants to the encapsulation solution, the ratio of encapsulation gel to liquid, and others factors that would be familiar to those practiced in the art upon reading this description. The efficiency of cell production could be affected by the timing of the emerging EB, as once the EB emerges from the capsule, that particular method of prevention of EB aggregation would be gone. The effect of any subsequent EB aggregation would depend on the stage of development of the emerged EB and any limitations in size (numbers of cells) of an EB. The size of the capsule could also effect efficiency of delivery of various growth or differentiation factors or any other factors or nutrients to the cells. For instance larger capsules would have greater surface in which such factors could pass through, or provide a substrate onto which one could functionalize bioactive molecules (Irvine et al, 1998). This could also be accomplished by controlling the porosity of the encapsulation matrix. In one embodiment, the method of the invention enables the culture of differentiating ES cells in conventional commercially available bioreactor systems that include the use of standard bioprocess equipment (probes, filters, etc).

The matrix of the capsule can be composed of a variety of substances. In one embodiment it is an agarose capsule. In another embodiment, the matrix should be biocompatible in that it should not have a detrimental effect on cell proliferation and/differentiation as desired. In yet another embodiment, the matrix should such that permits the passage or exposure of desired media components to the spheroid-forming cells or spheroid. This can be done by keeping certain components in the capsule and others out or by permitting the

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passage of certain components through the capsule matrix or by including certain components in the capsule matrix.

The encapsulation material chosen should prevent aggregation of cells contained in different capsules. Suitable material would include but is not limited to agarose, alginate, polymers such as poly HEMMA (Valbacka et al (2001) and others or matrices described in Weaver et al. (1988) and Sefton et al (1977).

The material selected for encapsulation should permit adequate delivery of any desired nutrients, cell culture media, growth factors, cytokines, or any other factors to promote desired spheroid-forming or ES cell growth, proliferation, differentiation or spheroid or EB formation, e.g., through functionalization of the encapsulation matrix, or by diffusion through the pores. The culture media can also include other factors such as low molecular weight molecules or drugs, such as retinoic acid or DMSO, as desired. Factors can also be included in the media for testing their effect on the cells. The material selected may depend on the factors that are to be delivered. Example of matrices that could be used are agarose, alginate, and polymers that support cell growth. Examples of suitable matrices are described in Weaver et al. (1988) and Sefton et al (1977). However, a person skilled in the art would be familiar with other suitable matrices upon reading this description.

Examples of culturing conditions that promote spheroid-forming cell or ES cell differentiation and spheroid or EB formation include but are not limited to conditions described in Keller et al. 1995 or O'Shea et al. 1999 or those outlined in Tables 10 or 11 and references noted therein to enhance generation of particular cell types. The spheroid-forming cell and other cells used in the invention can be genetically modified cells. The genetic modification can be for a variety of purposes including, to enhance cell selection by conferring particular identifying characteristic to the desired cell, to generate cells for gene or cell therapy, transplantation (for instance to reduce or prevent graft versus host disease and/or rejection) diagnostic and/or screening purposes. Other applications would be apparent to those skilled in the art.

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More particularly, the conditions under which the ES cells are cultured for differentiation will depend on the type of ES cell derived cells and tissues desired. For instance, to obtain hematopoietic stem cells one would preferably culture the ES cells under certain conditions, such as described in Wiles and
5 Johansson 1999; Rathjen et al. 1998; or Keller et al, 1993. On the other hand, if one wishes to obtain cardiomyocytes, the cells would be cultured under specific conditions, such as in the presence of specific cytokines, i.e. TGF- β (Dickson et al. 1993), or using a selectable genetic marker such as described for cardiac myocytes in Klug et al (1996). In vitro, ES cells have been shown
10 to differentiate into a variety of cell types and tissues, including beta cells, cardiomyocytes, hepatic cells, kidney cells, neuronal cells, and hematopoietic cells. Various cell types and culturing conditions are described in Zandstra and Nagy, 2001 for general approaches; Wang et al, 1992 for endothelial cells; Rohwedel et al., 1994, Fleishman et al, 1998 and Mummery et al. 2002
15 for cardiac and muscle cells; Bain et al, 1995, Tropepe et al, 1999 and Reubinoff et al. 2001 for neuronal cells; Palacios et al, 1995 and Kyba et al., 2002 for hematopoietic stem cells; Choi et al, 1998 and Levenberg et al. 2002 for hematopoietic and endothelial cells. Assady et al. 2001 for insulin – producing cells. For other examples of culture conditions see Tables 10 and
20 11. Since the differentiation of the ES cells results in a complex mixture of all possible cell types an efficient enrichment protocol would be necessary to obtain a homogeneous population of specific cell types or tissues to be utilized for various purposes including the generation of surrogate cells for human therapies or for drug screen testing. This enrichment could be done by
25 the introduction of a specific construct into ES cells, comprising a cell or tissue type specific promoter controlling the expression of a marker gene. The resulting transgenic ES cell(s) can be expanded, differentiated and selected to generate the desired cell type of interest using techniques known in the art, for instance as described in Klug et al (1996) for cardiac cells (WO 95/14079,
30 26 May 1995; U.S. Patent Nos. 5,733,727 and 6,015,671) or Soria et al (2000) or Soria et al (2001). The method is widely applicable to any cell type for which the tissue specific promoter is known. Table 9 provides some

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examples of promoters useful for the selection of specific cell lineages without limiting the number of useful promoters to that table.

Particular cell types can be selected for and/or harvested using a variety of methods, including cell surface receptors or other labeling, tagging or monitoring or selection methods known in the art, such as expression of particular types of genes, proteins or levels of certain molecules.

In a further embodiment, the invention provides a method for the selection of specific cell types from spheroid-forming cells comprising the steps of:

- i. introducing a reporter gene expressing vector into at least one spheroid-forming cell whereby a cell-type specific promoter is combined with an reporter gene, such that the reporter gene is expressed under the control of the cell-type specific promoter;
- ii. culturing the spheroid-forming cell(s) , for example to expand the cells
- iii. differentiating spheroid-forming cell(s), for instance in accordance with the method of the present invention under controlled aggregation conditions.
- iv. isolating and harvesting the specific cell-type based on the reporter gene expression

In one embodiment, the reporter gene is an antibiotic resistance gene and the cell-type of interest is isolated by the addition of an appropriate antibiotic in step (iii) or (iv).

In yet another embodiment, the method for the selection according to the reporter gene is selected from the group consisting of the Hygro mycin resistance gene (hph), the Zeocin resistance gene (Sh ble), the Puromycin resistance gene (pacA), and the Gentamycin of G418 resistance gene (aph).

In yet another embodiment, in the method for the selection the reporter gene is selected from the group consisting of luciferase, green fluorescence protein, red fluorescence protein, and yellow fluorescence protein and the cells of interest are selected by fluorescent activated cell sorting (FACS).

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In yet another embodiment, in the method for the selection the reporter gene is selected from the group consisting of luciferase, green fluorescence protein, red fluorescence protein, yellow fluorescence protein, or a his-, myc-, or flag-tag ligated to a heterologous gene, or any heterologous gene which
5 when expressed is inserted into the cell surface and the cells of interest is isolated from the cultured cells by affinity purification.

In yet another embodiment, in the method for the selection, the cell type specific promoter is selected from those listed in Table 9 for cell types listed therein.

10 The initial undifferentiated spheroid-forming or ES cells used in the method can be obtained by methods known in the art. For instance one can use ES cells obtained directly from the inner cell mass of the embryo, or one can use ES cells that were cultured, i.e. expanded under conditions that preferably did not promote differentiation, such as described in Klug et al.
15 1996.

In another embodiment the method of the invention enables culture of differentiating spheroid-forming or ES cells in stirred 3-dimensional reactors with scalable volume.

20

In another aspect the invention provides a culture bioreactor for industrial production of cells derived from spheroid-forming cells comprising:

- a. culturing spheroid-forming cells in accordance with the method of the invention under controlled aggregation, wherein the cells
25 are cultured in a spheroid-forming cell suitable media under conditions that promote spheroid formation; while
- b. inhibiting or preventing spheroid aggregation.

Such suitable media conditions are any conditions that promote spheroid formation, examples of which are provided herein.

30 In another embodiment, the invention provides a bioreactor for generating cells from spheroid-forming cells comprising a means for controlling conditions suitable for spheroid formation, such conditions comprising a means for controlling cellular aggregation and means for

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maintaining said cells and generated cells in suspension, such as means for preventing cellular aggregation.

5 In yet another embodiment the invention allows for measurement and control of the culture environment by enabling the use of stirred or other well-mixed bioreactors.

As a result of being able to better control the ES cell culture environment, the method of the invention enables one to better study various conditions to obtain particular differentiated ES cells and tissue (i.e. hematopoietic cells, cardiomyocytes, nerve cells, beta cells, hepatic cells, 10 kidney cells, any other cell types differentiated or not, known to develop from embryonic stem cells) and to better optimize these conditions. As such, the method of the invention can be used to derive a cell culture with a higher density of desired differentiated cells. The ability to do this may determine whether or not the process is economically viable and therefore is critical to 15 the translation of these technologies from the lab scale.

The method of the invention can also be used to identify factors (e.g., any variant, such as growth factors, differentiating factors, such as cytokines, or other conditions, such as pH, temperature, oxygen or factor concentration levels) and conditions that effect and preferably optimize spheroid-forming cell 20 or ES cell differentiation and spheroid or EB formation. Examples of some factors, especially for studying spheroid-forming cell or ES cell differentiation to cardiomyocytes include but are not limited to fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), endothelin-1(ET-1), stem cell factor (SF), 25 opiod peptide (or supplemental DMSO), bone morphogenic protein (BMP) family members transforming growth factor beta (TGF-beta), retinoic acid (RA). Factors preferably used to study beta-cell differentiation include but are not limited to, glucose levels, nicotinamide, KGF (karatinocyte growth factor), EGF (epithelial growth factor, NGF (neural growth factor) and TGF-beta, the 30 above factors, as well as others typically used on adult hematopoietic stem cells (Zandstra et al 1997) can also be used to elicit the development of hematopoietic and endothelial cells from pluripotent cells. This method would comprise culturing the spheroid-forming cells or ES cells under conditions that

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control cell aggregation in the presence of the particular factor to be tested and then detecting the effect of the variant on cell proliferation and differentiation. The effect of various different concentrations or formulations of the factor can also be tested. Preferably a negative control is used, whereby
5 the spheroid-forming cells or ES cells are cultured in the absence of the factor to be tested.

The methods and products of the invention can also be used to produce cells useful for drug therapy testing, to identify targets for gene and cell therapy, and in assays for such methods.

10 Spheroid-forming cells, such as ES cells and the cells and tissues derived therefrom can also be used in the treatment of various cancers, leukemias, autoimmune diseases, organ failure, animal or tissue cloning, gene therapy, transgenic animals.

The spheroid-forming cells and ES cell-derived cells and tissues
15 obtained from the invention can be used to provide cells and tissues for transplantation. For instance, ES cells can be grown under conditions to produce hematopoietic cells. Such cells could be used in bone marrow transplants, blood transfusions or infusions. ES cell derived cardiomyocytes can be used in tissue engineering, cell/tissue transplantation, gene therapy,
20 and drug discovery. ES cells derived skin tissue can be used for reconstructive surgery, ie., for burn victims, cosmetic surgery to name a few.

Further examples of applications for ES derived cells and tissues can be found in Rathjen et al 1998 or Marshall E. 2000.

In yet another embodiment the invention is directed to a method to
25 identify factors (any substance, media or other conditions, for instance oxygen levels, pH, pressure, cell density) that effect cell proliferation, differentiation and/or spheroid formation or other cellular or spheroid characteristic desired to be monitored, said method comprising culturing the cells as per the method of the invention in the presence of the factor to be tested and then monitoring
30 the effect of the factor on cell proliferation, differentiation and/or spheroid formation or other cell or spheroid characteristic desired. Such monitoring can include comparing the effect of the factor on cells in light of a control culture. In one embodiment, such a control culture can be a negative control

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which does not contain the factor to be tested. Such factors can include factors for screening compounds that may be useful for the treatment or diagnosis of various medical conditions.

5 It should be noted that although the discussion above describes the invention in terms of ES cells, the same methods and applications and products of the invention would be applicable to pluripotent cells in general or spheroid-forming cells other than ES cells.

The present invention also includes the Spheroid-forming and Embryonic stem cell derived cell culture obtained using the method of the
10 invention, including the initial encapsulated, and/or high density cell culture

In another embodiment the invention provides a method to prevent aggregation between cells comprising encapsulating a cell or group of cells and thus preventing aggregation of said cells with cells not within said capsule. Further the invention also encompassed the use of encapsulating
15 cells, as described herein to control the micro environment of a cell to be cultured. This is not limited to spheroid-forming or ES cells. Such methods can be used to proliferate such cells or to control culturing conditions. To optimize cell culture conditions and to identify factors that effect culture conditions. A liquid suspension comprising encapsulated cells, such as
20 spheroid-forming cells and derived cultures there from are also encompassed within the scope of the present invention.

The following non-limiting examples are illustrative of the present invention:

25 EXAMPLES

Materials and Methods

ES Cell Culture

Murine embryonic stem cells: CCE, R1, D3 (mentioned below) and
30 others (Beddington and Robertson, 1989; Nagy, Rossant et al., 1993), were maintained at 37°C in humidified air with 5% CO₂ in Iscoves Modified Dulbecco's Medium (IMDM, GIBCO-BRL) supplemented with 15% fetal bovine serum (Hyclone), 50 U/ml penicillin (GIBCO-BRL), 50 µg streptomycin

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(GIBCO-BRL), 2mM L-glutamine (GIBCO-BRL), 0.1 mM 2-mercaptoethanol, and 500 pM leukemia inhibitory factor (LIF) (ES cell media). Culture flasks were prepared prior to cell seeding by coating with a solution of 0.1% porcine gelatin in phosphate buffered saline. All ES cells were used between passages 15-24.

ES cell differentiation systems

ES cells were dissociated using 0.25% trypsin-EDTA (Sigma) and cultured in ES cell medium without LIF to induce differentiation. Liquid suspension cultures (LSC) were prepared by aliquoting 10 ml of cell solution at the indicated ES cell density into 10 cm petri-dishes (Fisher). For single ES cell analysis (single cell liquid suspension culture scLSC), a single ES cell was placed into each well of a 96 well plate (Nunc) coated with 10% pluronic (F-127, Sigma) solution to prevent cell attachment. This was achieved by preparing a suspension of 20 ES cells/ml and aliquoting 50 μ L into each well. Each well was then visually inspected for the actual number of deposited cells. Empty wells or wells with more than one cell were marked and not included in the analysis. Methylcellulose cultures (MC) were prepared by aliquoting 1 ml of cell suspension with 1% methylcellulose (M3220, Stem Cell Technologies) at the indicated ES cell density in 35mm Greiner dishes (Greiner Labortechnik). Hanging drop cultures (HD) were prepared using 10 μ L droplets, each containing the number of ES cells desired to initiate the EB (as indicated). ES cells were allowed to aggregate in hanging drops for two days before transfer to liquid suspension culture.

Stirred cultures were performed in spinner cultures (Bellco Glass) ranging from 50 to 250ml of media/vessel. ES cells were added to the media directly (stirred cultures without encapsulation: SC) or encapsulated (ESC) as indicated. A paddle-style impeller was used to agitate the media at 40-60 RPM.

EBs were enumerated by visual inspection. EBs were dissociated by incubation (1 min, 37°C) in 0.25% trypsin-EDTA (Sigma), or incubation (30 min, 37°C) in 0.25% collagenase (Sigma) in PBS supplemented with 20% fetal bovine serum for post day 7 EBs. Mechanical shearing was also used to

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help dissociate cells into a single cell suspension by twice passaging the cell mixture through a 21-gauge needle and syringe (Becton Dickinson). Individual cells were then counted using a hemocytometer and analysed by either myeloid-erythroid colony-forming cell (CFC) assay (Zandstra et al. 1997) or flow cytometry (Zandstra et al. 1997)

Gel Microdrop (GMD) Encapsulation of Individual ES Cells

Individual CCE ES cells were suspended in Hanks Buffered Saline Solution (HBSS) at a concentration of 10^7 cells/ml and added to a 3% agarose solution for a final cell concentration of 2×10^6 cells/ml. The agarose solution was dispensed into mineral oil (nonsolvent) at 37°C and subjected to impeller blending using the One Cell System (Ryan et al., 1995) to create agarose microdrops containing individual ES cells. The GMDs were washed twice with HBSS to remove the mineral oil before resuspension in ES cell media without LIF for differentiation culture.

Gel Microdrop(GMD) Encapsulation of Multiple ES Cells

Three different methods were used to encapsulate multiple ES cells.

(1) ES cells were gently dissociated from adhered culture using trypsin, creating multi-ES cell clumps or aggregates. Clumps were subsequently encapsulated as described for individual ES cells.

(2) ES cells were dissociated to a single cell suspension and cultured at high cell density ($>10^5$ cells/ml) in unagitated petri dishes for 12-24 hours to induce cell clump formation via controlled aggregation. The average number of ES cells per clump was controlled by input cell density and by time, and was determined by dividing the input number of ES cells by the number of clumps counted. ES cell clumps were then encapsulated as described.

(3) Individual ES cells were mixed with agarose at a concentration of $1-5 \times 10^7$ cells/ml and encapsulated as described. Capsules formed in this way were essentially clumps of individual ES cell held together by agarose matrix. Capsules themselves were considered ES cell clumps and again encapsulated as described.

Hematopoietic Colony Forming Cell Assay

Cells were plated into methylcellulose medium (M3434, Stem Cell Technologies) containing 50 ng/ml mrSCF, 10 ng/ml mrIL-3, 10 ng/ml hrIL-6,
5 3 U/ml hEpo, 15% fetal calf serum, 0.1 mM 2-mercaptoethanol, and 2mM L-glutamine. Hematopoietic colonies were scored by morphology after 6-7 days.

Generation of cardiac myocytes from ES cells

10 CCE and R1 embryonic stem cell were transfected with the MHC-neo^r/PGK-hygro^r DNA as previously described (Klug et al 1996, WO 95/14079, 26 May 1995; U.S. Patent Nos. 5,733,727 and 6,015,671). Briefly, 1 µg of DNA and 25 µg of salmon testes DNA were mixed in a total volume of 800 µl of cells suspended at 4×10^6 cells / mL on ice. Cells were
15 electroporated (180 volts, 80 mF), incubated on ice for 15 minutes, and plated in 100 mm corning dishes in ES media containing 500pM LIF. Cells containing the plasmid were selected by culture in ES medium with LIF and 200 µg/mL hygromycin B for 7 days with daily medium exchange. Test ES cells were then encapsulated using the multiple ES cell encapsulation
20 technique as described above in the third technique. After 8-10 days of culture in stirred suspension bioreactors (Bellico spinner flasks or Applikon 1L cell culture bioreactors) in ES cell media without LIF, the G418 was added to the medium to select for the cardiac cells. The composition of the selected cells was analyzed by MF-20 staining using flow cytometry. 5×10^5 - 10^6 cells to be
25 stained for MF-20 were washed with PBS and fixed with 100 µl of IntraPrepTM Permeabilization Reagent 1 (Immunotech, Westbrook, ME) for 15 minutes at room temperature. After washing once with 1 ml PBS, the cells were permeabilized for 5 minutes with IntraPrepTM Permeabilization Reagent 2 and incubated with a 1:10 dilution of the mouse anti-sarcomeric myosin heavy
30 chain (MF-20) antibody (Transduction Lab, MA) for 15 minutes at room temperature. After washing once with PBS, secondary FITC-conjugated goat anti-mouse IgG antibody was added with 1:100 dilution and the cells were incubated for 15 min at room temperature. In addition, some of the dispersed

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cells were used for determining the viability of the cells immediately after the procedure of enzymatic dissociation. Test cell populations were washed in ice-cold Hanks hepes-buffered salt solution containing 2% FCS (HF) and resuspended at 10^6 cells/100 μ L in HF. Finally, the cells were analyzed on a flow cytometer (XL, Beckman-Coulter, Miami, FL) using the EXpoADCXL4 software (Beckman-Coulter). Positive staining was defined as the emission of a level of fluorescence that exceeded levels obtained by >99.9% of cells from the control population stained with only the secondary antibody. Cardiac development was also determined by electron microscopy.

Generation of Hematopoietic and Endothelial Cells

Hematopoietic and endothelial cells were generated in through ES cell differentiation in ES cell media without LIF under the conditions described in Dang et al. 2002. Input ES cells were encapsulated using the multiple ES cell encapsulation technique as described above in the third technique.

Flow Cytometry for analysis of endothelial and hematopoietic cells

Cells were washed in ice cold Hanks hepes-buffered salt solution containing 2% FCS (HF) and resuspended for 10 min at 4°C in HF containing α -mouse CD16/CD32 monoclonal antibody (PharMingen) at 1 μ g/100 μ L to block non-specific binding. Blocked cells were then incubated at 10^7 cells/ml for 40 min at 4°C with FITC anti-mouse CD34 (PharMingen), PE anti-mouse Flk1 (PharMingen), and anti-mouse E-Cadherin (Sigma). Anti-mouse E-Cadherin incubation was followed by two washes in ice cold HF and then incubated for 40 min at 4°C with anti-mouse FITC-conjugated IgG (Sigma). Stained cells were washed twice in ice cold HF, and 1 μ g/ml 7-amino-actinomycin D (7AAD, Molecular Probes) was added to the final wash. The cells were analyzed on Coulter Epics XL using 4-Colour Expo 32 software for analysis. Positive staining was defined as the emission of a level of fluorescence that exceeded levels obtained by >99.9% of the cells from the same starting population stained with a matched fluorochrome-labelled irrelevant isotype control antibody.

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Measurement of Media composition

Oxygen tension, in addition to other parameters, was measured by withdrawing 3ml of culture media using a gastight syringe (Hamilton), and analyzing using a BioProfile 200 blood/gas analyzer (Nova Biomedical).

5

Neurosphere formation

R1 murine embryonic stem cells were dissociated into a single cell suspension and transferred at 10^4 ES cells/ml to media comprised of Dulbecco's Modified Eagle Medium (DMEM, GIBCO-BRL) supplemented with 20% Knockout serum replacement (Sigma), 50 U/ml penicillin (GIBCO-BRL), 50 μ g streptomycin (GIBCO-BRL), 2mM L-glutamine (GIBCO-BRL), and 0.1 mM 2-mercaptoethanol.

10

Example 1 - Effects of Cell-Cell Adhesion and EB Aggregation on ES cell culture

15

To assist in the development of a new bioprocess, the properties of ES cells and EBs that might affect bioprocess efficiency, net cell expansion, and cell fate decisions were investigated. EB formation, growth and development between different culture techniques were compared. Once an understanding of these basic parameters was obtained, guidelines to the design of efficient, scalable bioprocesses were developed.

20

(a) Some ES cell lines can form EBs from individual cells.

Preliminary studies focused on establishing the *in vitro* developmental potential of individual ES cells. In single cell liquid suspension (scLSC) and methylcellulose culture (MC), EB efficiency (number of EBs divided by input number of ES cells) have been reported anywhere between 2-15%. Because these values were so low, it was uncertain as to whether an individual ES cell could form an EB, or if EB formation occurred only when ES cells aggregated by chance (or by design, as in the hanging drop method). Therefore the first constraint investigated was the number of ES cells required to form an EB.

30

Individual murine CCE ES cells were placed by limiting dilution into separate wells of a 96 well plate and differentiated in liquid suspension culture

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(ES media without LIF). One hour after plating, each well was visually examined to verify the number of cells plated. After seven days of differentiation, the wells were examined for cell aggregates. The cell aggregates were identified as EBs based on their expression of Flk-1 and CD34, and their ability to form hematopoietic colonies (data not shown). By dividing the total number of EBs per 96 well plate by the number of input ES cells, we determined that individual ES cells were able to form EBs with an overall efficiency of at least $42 \pm 9\%$ (Table 1). In a separate set of experiments, it was determined that $78 \pm 9\%$ of the starting cell population were undifferentiated ES cells based on E-cadherin and SSEA-1 expression (data not shown, (Zandstra, et al. 2000)). Viability, based on exclusion of dead cell dye 7AAD, of the ES cell population after trypsinization was determined to be $87 \pm 11\%$. This allowed recalculation of the EB formation efficiency from individual, undifferentiated, and viable ES cells to be approximately 60%. These results clearly demonstrated that a majority of individual murine CCE ES cells have the capacity to form an EB, and that these cells could form EBs at a very high efficiency. However, the results also raise the issue of why such high efficiencies were not regularly achieved in standard differentiation cultures.

The present inventors have shown that murine CCE ES cells have demonstrated the ability to form EBs from single ES cells. In other cell types or under other conditions contact between multiple ES cells to enable EB formation may be preferred. For example, the present study repeated with single murine R1 and D3 ES cells did not form single ES cell derived EBs under the conditions used. Instead, aggregation with approximately 5 or more ES cells was required for survival, proliferation and differentiation (see below). Similarly, it has been reported [Thomson JA, Odorico JS. 2000] that current human ES cell lines form EBs only from multi-ES cell aggregates or clumps. Regardless, the following aggregation studies reported with CCE ES cells are still illustrative of the extent of EB aggregation and the problem it poses. As such controlling cellular aggregation during expansion and differentiation of said cells is an important factor.

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(b) EBs aggregate, reducing EB formation efficiency

Further investigation revealed the sensitivity of EB efficiency to initial ES cell concentration and time (Figure 3). Various initial CCE ES cell concentrations were differentiated in liquid suspension and methylcellulose cultures, and the number of cell aggregates (EBs) per dish was recorded at regular time intervals. In liquid suspension culture, EB formation efficiency increased as ES cell concentration decreased (Figure 3A). Over time, EB formation efficiency decreased for all cell concentrations. However the extent of this decrease was most dramatic in cultures initiated with 10^5 ES cells/ml, and almost nonexistent at 10^2 ES cells/ml. In methylcellulose cultures (Figure 3B) initiated with 10^3 ES cells/ml or less, EB formation efficiency remained constant at approximately 40% over the duration of the experiment. EB formation efficiency gradually decreased in methylcellulose cultures initiated with 10^4 or more ES cells/ml, however to a lesser extent than their liquid suspension counterparts.

Based on their similarity, the model of the present invention for EB formation from single ES cells ($42 \pm 9\%$) was reconciled with EB efficiency in methylcellulose culture initiated with 10^3 ES cells/ml or less ($40\% \pm 6\%$) and liquid suspension culture initiated with 10^2 ES cells/ml or less ($34 \pm 5\%$). However, an explanation was still required for the discrepancy with the results obtained at higher cell concentrations. It was recognized that the decrease in EB efficiency over time indicated that the process of EB formation itself was unaffected; rather, another process occurring post-EB formation must be reducing the number of EBs present. Observing the cultures, it was apparent that EB aggregation was the cause of inefficiency. In this process, separate EBs aggregated and merged into a single, larger EB.

This phenomenon accounts for decreased EB efficiency with increased cell concentration since likelihood of EB collision and subsequent aggregation would also be greater. Furthermore, aggregation explains the higher EB efficiency in methylcellulose cultures compared to liquid suspension culture: Semi-solid methylcellulose media impaired cell movement, thereby decreasing likelihood of EB collision and aggregation.

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(c) Aggregation reduces net cell expansion

The results demonstrate that CCE ES cells form EBs with an efficiency of $42 \pm 9\%$, a value independent of culture method and initial ES cell concentration, and that EB aggregation reduced the apparent efficiency over time. To determine what effect EB aggregation might have on the overall production of cells, the growth rate of various initial ES cell aggregate sizes (1, 100, and 1000 ES cells/EB) formed using the single cell liquid suspension culture (scLSC) and hanging drop techniques were compared (Figure 4, Table 2). Regardless of initial aggregate size, all EBs contained a similar number of cells by day 12. Since final EB size is independent of EB aggregation, EB aggregation reduces overall expansion of cells.

(d) Cell-cell adhesion molecules are responsible for EB aggregation

The process of EB aggregation was studied using the simplest scenario: aggregation of two EBs. To visualize cell mixing, one EB generated from wildtype ES cells was aggregated with one EB generated from ES cells constitutively expressing GFP (Hadjantonakis et al 1998) . The extent of aggregation was quantitatively determined by the "degree of aggregation" (DOA) - the ratio of the diameter of the interface to the overall length of the two EB system (Figure 5). Regardless of initial aggregate size (50, 100, 400 ES cells/EB, data not shown), complete fusion (DOA > 90%) was achieved after approximately 16 hours and complete cell mixing (homogenous fluorescence intensity) after 48 hours (Figure 5).

The mechanism of EB aggregation was investigated using various loss of function treatments (Figure 6). Blocking surface cell adhesion molecules with blocking peptide inhibited EB aggregation. E-cadherin blocking was particularly efficient, also noted by Larue et al. (1996) who observed that homozygous E-cadherin null ES cells were unable to aggregate as clumps. EBs treated with cytochalasin D also were unable to aggregate. Cytochalasin D inhibits actin-dependent processes including cell migration and cell division. To be sure that impaired cell division was not responsible for this effect, another group was treated with Mitomycin C that inhibited mitosis by preventing DNA synthesis. These EBs aggregated at the same rate as the

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control (no treatment). Based on these results, a two-step mechanism for EB aggregation was proposed: First, neighboring EBs collide and homophilic E-cadherin molecules adhere, holding the EBs together. Cells then migrate and remodel as the EBs fuse into a single spheroid.

5 Preventing cell adhesion versus cell migration between neighboring EBs was the logical strategy for preventing EB aggregation as treatments that could affect cell growth or differentiation (i.e. cytochalasin D) would be undesirable for most applications. E-cadherin, the prominent cell adhesion molecule in this process, is a Ca^{2+} -dependent homophilic cell-cell adhesion
10 molecule expressed by ES cells. As ES cells differentiate, E-cadherin expression is downregulated (Zandstra et al. 2000). Using flow cytometry, E-cadherin expression was tracked in differentiating EBs over time (Figure 7.A). Expression decreased as cells differentiated - from almost 100% on day 0 (ES cells) to approximately 25% by day 5 of differentiation. As expected, E-
15 cadherin expression correlated with rate of EB aggregation: EBs differentiated for four days or fewer aggregated within 16 hours, while day 6 EBs did not aggregate (Figure 7.B). Similarly, while ES cells placed into stirred culture quickly aggregated into large cell clumps that did not grow or differentiate properly, EBs grown in static culture for a minimum of four days could be
20 transferred to stirred culture with little aggregation. These EBs continued to grow and differentiate normally. Therefore, the strategy adopted for controlling cell adhesion need only be implemented while cell adhesion molecule expression remains high. For murine ES cells, this time period is the first four days of differentiation.

25 These results suggest that novel approaches to control the aggregation of undifferentiated ES cells and their derivatives may allow them to be cultured in high cell density, stirred suspension bioreactors.

30 **Example 2 - Preventing ES Cell-Cell Adhesion and EB Aggregation**
Results in Efficient Generation of Embryonic Stem Cell Derived Hematopoietic and endothelial cells

The overall schematic of one embodiment of the bioprocess of the invention for the scalable culture of ES cell derived tissues is shown in Figure

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2. First, undifferentiated ES cells are expanded under appropriate conditions. ES cells are then cultured in a bioreactor conditions that control aggregation and promote cell proliferation and differentiation. More specifically, aggregation of ES cells to induce EB formation is permitted and aggregation between cells within an EB is permitted. However aggregation between ES cells beyond that required to induce EB formation is prevented, and aggregation between separate EBs is prevented.

After an appropriate period of culture, under defined conditions (including growth factor and medium formulation, oxygen tension, pH, medium supplementation rates), differentiated cells are selected for and used in clinical or pharmaceutical applications. In one aspect, the present invention is primarily concerned the scalable generation of ES cell derived cells and tissues.

15 **(a) Culture of EB Under Non-Aggregating Conditions**
Genetically modified ES cells

E-cadherin⁻ ES cells are defective in cell adhesion, unable to grow as clumps or aggregates of cells as they typically do (Larue, Antos et al. 1996).

20 *Antibodies to block cell adhesion molecules*

As reported in the present study of EB aggregation, treatment of EBs with E-cadherin blocking peptides inhibits EB aggregation. Blocking peptides effectively nullifies the function of E-cadherin, as reported by Burdsal et al. (1993). Blocking E-cadherin prevents EBs from adhering with neighboring EBs, thus circumventing subsequent aggregation.

Reducing inorganic salt concentration or inhibition of ion channels

Cell adhesion can be inhibited by substantially reducing inorganic salt concentration (Boraston et al. 1992). For example, cell adhesion modulated by calcium-dependent adhesion molecules such as cadherins depends upon calcium influx from both extracellular calcium through calcium channels and release from internal calcium stores. Calcium channel inhibitor LaCl₃ or thapsigargin was shown to inhibit cell-cell attachment (Ko, Arora et al. 2001).

(b) Encapsulation of ES cells

Preventing EB aggregation was the guiding design principle for new bioprocesses capable of efficient and scalable differentiation of ES cells.

5 While it was suspected that E-cadherin was the main molecule involved in cell aggregation, a method was developed to preclude interactions between all cell-cell adhesion molecules on the EB surface by physically blocking their association. In this method, ES cells are encapsulated within a biodegradable matrix and allowed to grow and differentiate in liquid culture, such that each

10 cell-containing capsule gives rise to a single EB. The surrounding matrix prevents separate EBs from contacting one another, thereby preventing aggregation. Formation of EBs in this novel way permits high cell density culture in a homogeneous, controllable, and scalable bioreactor system. The bioprocess of the present invention is termed: encapsulated stirred culture

15 (ESC). The first step in this method involves the encapsulation of individual or multiple ES cells. Uniform or "synchronized" EB sizes could be achieved by encapsulating similar numbers of ES cells per capsule. The matrix could be agarose, alginate, or another type of natural or artificial matrix (e.g. polymer) that does not interfere with the growth and differentiation of EBs. Depending

20 upon the matrix used, cells might degrade the matrix as they grow (as is the case with agarose), or they might burst the capsule as cell growth places increasing pressure on the capsule (as is the case with alginate), or they can be manually released from their capsules by adding degradation enzymes (such as agarase addition to agarose), or changing the culture conditions

25 (such as depleting dissolved calcium ions in the case with alginate). Therefore, the size and composition of the capsule can determine the amount of time the EB remains encapsulated. For example, agarose is a simple polysaccharide readily degraded by the cells as they grow. Therefore, the diameter of the agarose capsules determines the amount of time the EB

30 remains encapsulated (Figure 8.A, Table 3). Capsules are designed to retain the EB until cell differentiation results in downregulation of E-cadherin (or other cell adhesion molecule) expression. After this time, EBs that emerge from the capsules will not aggregate. For differentiating EBs from murine ES

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cells, E-cadherin is sharply down regulated after 4 days (Figure 7.A). It was determined that 70-100 μm agarose capsule diameters provide adequate protection for this period of time, and such capsules were specifically designed (Figure 8.B). However, it should be understood that the invention is not intended to be limited to such a capsule size as a person skilled in the art would appreciate that other sizes would work.

(c) Encapsulation enables use of stirred culture

ES cells placed directly into stirred culture quickly aggregate into large cell clumps that did not proliferate or differentiate into any of the cell types assayed for (data not shown). Encapsulating ES cells prior to their introduction to stirred culture enabled efficient EB formation and prevented their aggregation. EB efficiency between liquid suspension culture (LSC) [non-stirred, non-encapsulated], stirred culture (SC), and encapsulated stirred culture (ESC) was compared at 10^3 and 10^4 input ES cells/ml after 7 days of differentiation culture (Table 4). Whereas EB efficiency in LSC declined as ES cell density increased, EB efficiency remained high in ESC.

Fold expansion of total cells in liquid culture (LSC), stirred culture (SC), and encapsulated stirred culture (ESC) is illustrated in Table 5. Cultures were initiated with 10^4 R1 ES cells/ml and analyzed after 7 days of differentiation. Table 5 shows results of fold expansion, after adjusting for cell loss during encapsulation procedure. Yield of encapsulated ES cells was approximately 25% of input. Cells were lost during the various transferring stages between centrifuge tubes, or retained in the mineral oil phase.

In stirred culture, EB efficiency was significantly improved with encapsulation than without. Encapsulated ES cells were able to form EBs that remained shielded from one another, thus maintaining a high EB efficiency by preventing EB aggregation. EBs emerged from the capsules after approximately four days - coinciding with down regulation of E-cadherin as previously described. Emerged EBs did not aggregate.

The ability to use stirred or other well-mixed systems ensure that bulk media conditions are homogenous, thus facilitating measurement and control

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of the system. These culture systems can also accommodate increase demand in cell generation by simply increasing the volume of the tank.

(d) Bioreactor does not significantly affect cell growth or differentiation

5 Hematopoietic development within EBs has been extensively studied and well characterized (Keller 1995). Hematopoietic development was therefore selected as the measure by which ES cell differentiation in different culture systems was compared. ES cells become blood cells through a series of developmental steps that can be tracked by cell surface marker expression
10 (Table 6). Murine ES cells differentiate into Flk-1 expressing mesoderm on day 4 that subsequently give rise to CD34 and CD45 expressing hematopoietic progenitor cells. Hematopoietic progenitors can also be assayed for using the hematopoietic colony forming cell (CFC) assay.

Ideally, the encapsulating matrix physically prevents EB aggregation
15 but does not impede transport of nutrients and metabolic products to and from the cells, nor influence differentiation or growth of EBs in any manner. To demonstrate this, ES cells were encapsulated in agarose and differentiated in static liquid culture. Cell proliferation and differentiation in this system was compared to standard ES cell differentiation systems: liquid suspension
20 culture, hanging drop, and methylcellulose cultures. Therefore agarose encapsulation of ES cells did not affect EB cell growth in any significant way.

Cell proliferation and differentiation was also compared between liquid culture (LSC) and the bioprocess enabled by the invention: encapsulated stirred culture (ESC) (Table 7). ESC was able to generate the hematopoietic
25 cell types following the same developmental kinetics as LSC. Cell proliferation and hematopoietic cell frequencies were comparable but not necessarily equivalent between the two systems.

EXAMPLE 3 - Use of Bioprocess to Generate Differentiated Cell Types

30 Generation of various differentiated cell populations was performed using encapsulated ES cells grown in stirred suspension bioreactors compared to LSC.

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a) Generation of hematopoietic cells from ES cells.

Mentioned previously, early mesoderm development was tracked by Flk-1 cell surface marker expression on day 4, while hematopoietic progenitors were detected phenotypically by co-expression of CD34 and CD45 cell surface markers, or functionally via methylcellulose colony assays. Using our novel invention, various types of hematopoietic progenitor cells were detected, from multipotent myeloid cells (CFC-Mix), to further restricted myeloid and erythroid cells (CFC-Ep, CFC-Ed, and CFC-GM) (Table 7). ES cells were differentiated in ES cell media in the absence of LIF. Because the culture was performed in a stirred bioreactor, it is feasibly scalable to any desired volume, and applicable to close microenvironmental control

b) Generation of endothelial cells from ES cells.

Using the same approach as described above (ES cell differentiated in ES cell media in the absence of LIF) in section a), endothelial cells were detected by coexpression of multiple endothelial cell markers: Flk-1, CD34, and CD31. A gate was drawn around Flk-1 and/or CD34 expressing cells, enabling exclusive analysis of gated cells for expression of CD31 (Figure 9). Because the culture was performed in a stirred bioreactor, it is feasibly scalable to any desired volume, and applicable to close microenvironmental control.

c) Generation of other cell types from ES cells

Other researchers have demonstrated that other cell lineages can also be generated from ES cells *in vitro*, including hepatocytes, β -cells, neurons, glial cells, kidney cells, muscle cells, endothelial cells, hematopoietic cells [i.e., Wang et al, 1992 for endothelial cells; Rohwedel et al., 1994, for cardiac and muscle cells 1994; Bain et al, 1995 for neuronal cells; Palacios et al, 1995 for hematopoietic stem cells; Choi et al, 1998 for hematopoietic and endothelial cells; Fleishman et al, 1998 for cardiac cells; Tropepe et al, 1999 for neural cells], and other cell types known to be derived from ES cells in the art. These cell types can be generated in stirred or mixed bioreactor cultures using media and general environment conditions as described in the prior art,

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only when aggregation is controlled, such as is possible using the bioprocess of this invention. This would be so as it was demonstrated that the bioprocess of the invention, e.g., encapsulation, does not adversely affect ES cell differentiation. The fact that this technology of the invention allows for the normal differentiation of ES cells, while facilitating the large-scale production of ES cell-derivatives makes it useful for a variety of cellular therapy-related applications.

Example 4 - The system facilitates measurement and control of culture environment.

Current ES cell differentiation processes are not amenable to measurement and control because the static and batch-style nature of these systems result in spatial and temporal gradients. In well-mixed (dynamic) systems, the bulk media conditions are the same everywhere at any given time. That is, any point measurement made within the system would be reflective of any other point. In this way, one can easily and accurately measure bulk conditions and also introduce control elements.

Using the bioreactor system of Example 2 above, EB development in normoxic (>160 mmHg O_2 tension) and hypoxic (30-40 mmHg O_2 tension) conditions was compared. Oxygen tension was measured offline using a blood gas analyzer and gas mixture in the incubator headspace was adjusting accordingly. Hematopoietic cell frequency, determined by CD34 and CD45 expression and hematopoietic CFC assay, was higher for EBs generated in hypoxic conditions (Table 8), indicating the positive effect that the ability to control and manipulate the microenvironment, such as allowed by this invention will have on the generation of cells and tissue from pluripotent cells.

The tendency for ES cells and early EBs to aggregate prevents the use of well-mixed bioreactor systems as well as higher cell density cultures. Therefore current differentiation systems are limited to static and batch-style conditions. The bioprocess of the invention enables ES cells to efficiently form EBs in well-mixed culture by controlling aggregation. As mentioned, the use of well-mixed systems automatically permits bulk media measurements and implementation of control strategies.

Example 5 – The Generation of Embryonic Stem Cell Derived Cardiac cells using ES cells Transfected with selectable markers

To demonstrate the improved ability to generate large numbers of clinically relevant cell types, the bioreactor culture of encapsulated ES cells was used to generate and select for cardiac myocytes. As for the above hematopoietic and endothelial development, flow cytometric analysis was employed to quantitatively evaluate the percentage of ES cell-derived cardiomyocytes generated during the course of differentiation in stirred bioreactors. In this case, ES cells transfected with the selection cassette as outlined above and described in detail in (Klug et al 1996, WO 95/14079, 26 May 1995; U.S. Patent Nos. 5,733,727 and 6,015,671) were cultured for 9 days in 500 mL stirred suspension bioreactors, prior to the addition of G418 to the media. At given time points the EBs were dissociated, the procedure previously described, resulting in a single cell suspension to be stained with a monoclonal antibody specific for sarcomeric myosin heavy chain (MF-20). Figure 10 Panel A shows kinetic changes of the percentage of the MF-20 positive cells during the course of differentiation and selection with G418 of transgene – containing ES cells cultured in stirred suspension bioreactors. Figure 10 panel B shows morphologic and structural analysis of bioreactor – grown ES cell-derived cardiomyocytes. The cells exhibit a mature sarcomeric organization and Z-banding (arrow). Figure 10 panel C shows a cross section of a "cardic body" (a spheroid of ES cell derived cardiac cells) generated in bioreactors.

Results showed that only a trace amount of MF-20 staining-positive cells was present in the EBs (0.7%) on day 0 after differentiation, however, the percentage of cells displaying positive staining with MF-20 rose gradually as a function of time after selection by G418, and reached over 70% at day 20 after differentiation. In contrast, the expression level of Oct-4, which is expressed in embryonic stem cells and is one of the first transcription factors differentially regulated during mouse development (Nichols and Zevnik, 1998) decreased significantly during the time course of differentiation, from 68.5% at day 0 to undetectable levels (at day 20 after differentiation (data not shown).

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The observation indicated that at the late state of differentiation and G418 selection, Oct4 positive ES cells were eliminated from the culture system. It is noteworthy that by day 15, the bioreactors typically contained between 2.5×10^7 to 1×10^8 viable cardiac myocytes (as assessed by MF20 expression). These observations were consistent in three independent experiments. In some experiments chemical factors such as retinoic acid were added to the media at specific time points to enhance cardiac differentiation and reduce the presence of undifferentiated ES cells (data not shown). The results indicate that differentiation of ES cells with the G418-selection system in spinner flask suspension culture system is capable of producing large quantity of cardiomyocytes.

Example 6: Generation of chimeric spheroids containing predetermined types of cells.

To show that the encapsulation system was capable of controlling the types of cells present in the spheroids at input, two populations of R1 ES cells, each labeled with a specific fluoro-chrome (green fluorescent protein, GFP) and (cyan) were encapsulated (see Hadjantonakis et al. 1998 and Hadjantonakis AK, Nagy 2001 for descriptions of the cells lines).

Materials and Methods

Individual R1 ES cells expressing the GFP protein by a constitutively active promoter (Hadjantonakis et al 1998) and individual trophoblast stem cells labelled with cyan as previously described (Tanaka et al. 1998) were suspended in Hanks Buffered Saline Solution (HBSS) at a ratio of 1 ES cell to 1 TS cell at a total cell concentration of 2×10^7 cells/ml and added to a 3% agarose solution for a final cell concentration of 4×10^6 cells/ml. The agarose solution was dispensed into mineral oil (nonsolvent) at 37°C and subjected to impeller blending using the One Cell System (Ryan et al., 1995). Microdrops containing a single ES cell and a single TS cell were identified and isolated by their side scatter characteristics, and by their GFP and cyan fluorescence using a FACSVantage Flow Cytometer (Becton Dickinson). Isolated microdrops were put sorted into ES cell media without LIF for analysis of their growth characteristics.

Example

To show that the encapsulation system was capable of controlling the types of cells present in the spheroids at input, ES cells and TS cells, each labelled with a specific fluoro-chrome (green fluorescent protein, GFP) and cyan were encapsulated as described. Using flow cytometry, the double positive population, (i.e., the microcapsules contained a single GFP cell and a single cyan labelled cells) were sorted into 96 well plates. Microscope based tracking of the cells confirmed that the two cell types survived and proliferated and formed spheroids containing two distinct sources of cells (chimeric spheroids). This technology provides the capacity to combine, in spheroids, two or more types of cells, including pluripotent cells and differentiated cells, for the end goal of manipulating the differentiation of the pluripotent cells into specific types of tissue using cell specific signals.

15 Example 7: Formation of ES cell derived neurospheres

ES cells were differentiated in serum-free conditions, yielding spheroids that did not stain for mesoderm markers. ES cells cultured in chemically defined serum-free, feeder layer-free, low-density culture conditions readily acquire a neural identity (Tropepe et al. 1999). Under these conditions, ES cells transition to a spheroid-forming primitive neural stem cell population capable of self-renewal and neural and non-neural potential - analogous to the neurosphere forming neural stem cells reported by Bjornson et al. (1999).

25 SUMMARY

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims. For instance, although ES cells were used in the Examples, the invention can be applied to other types of spheroid-forming or pluripotent cell types, such as embryonic germ cells and early primitive ectoderm-like cells or other type of

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stem cells such as neuronal stem cells or cells that can differentiate into other cell types and form spheroids *in vitro* during the differentiation process. Controlling aggregation during any type of cell expansion can also be beneficial in improving expansion efficiency of said cell type.

- 5 All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

TABLE 1

EB formation efficiency from CCE muES cells. A single ES cell was deposited into each well of a 96 well plate. Wells were visually inspected for EBs after 7 days. EB formation efficiency was determined by the ratio of EBs per 96 well plate, to the number of input ES cells per 96 well plate. Percentage of viable ES cells in the culture was determined by E-cadherin expression and exclusion of 7AAD dead cell stain.

Experiment Number	Input # of ES Cells	# of EBs	Actual EB Formation Efficiency	% E-Cad Positive Cells	Viability	% Viable ES Cells	Calculated EB Formation Efficiency
1	86	38	44.2%	68.5%	90%	61.7%	
2	97	33	34.0%	85%	75%	63.8%	
3	68	37	54.4%	80%	96%	76.8%	
4	70	26	37.1%	—	—	—	
Average	80.3	33.5	42%	78%	87%	68%	63%
Standard Deviation	13.8	5.4	9%	9%	11%	14%	17%

TABLE 2

Total cell expansion from EBs initiated with 1, 100, or 1000 cells/EB. Cell expansion improved significantly as fewer ES cells were used to initiate EB formation on day 0.

Initial Cells/EB (day 0)	Maximum Cells/EB	Expansion
1	17300 \pm 9900	17300
100	35100 \pm 11000	351
1000	35000 \pm 3000	35

TABLE 3

Effects of different encapsulation protocols on capsule diameter. 3 different parameters: impeller speed (revolutions per minute, RPM), time (minutes), and ambient temperature, are used to control capsule diameter.

Protocol step	35 μ m	50 μ m	85 μ m
1	2100 RPM 1 minute Room temp	1500 RPM 1 minute Room temp	1200 RPM 1 minute Room temp
2	2100 RPM 1 minute Ice bath	1500 RPM 1 minute Ice bath	1200 RPM 1 minute Ice bath
3	1100 RPM 10 minutes Ice bath	1100 RPM 5 minutes Ice bath	700 RPM 5 minutes Ice bath

TABLE 4

EB Efficiency in liquid culture (LSC), stirred culture (SC), and encapsulated stirred culture (ESC). Cultures were initiated with 10^3 or 10^4 CCE ES cells/ml and analyzed after 7 days of differentiation. EB efficiency in LSC declined as cell density increased. Aggregation occurred readily in SC regardless of cell concentration. Encapsulation effectively controlled cell aggregation, maintaining a high EB efficiency in stirred culture.

	LSC	SC	ESC
10^3 ES cell/ml	42±9%	<0.1%	41±21%
10^4 ES cells/ml	8.5±2.7%	<0.1%	35±3%

10

TABLE 5

Fold expansion of total cells in liquid culture (LSC), stirred culture (SC), and encapsulated stirred culture (ESC). Cultures were initiated with 10^4 R1 ES cells/ml and analyzed after 7 days of differentiation. The table shows results of fold expansion, after adjusting for cell loss during encapsulation procedure. Yield of encapsulated ES cells was approximately 25% of input. Cells were lost during the various transferring stages between centrifuge tubes, or retained in the mineral oil phase.

15

	LSC	SC	ESC
10^4 ES cells/ml	27±13	< 1	63±33

20

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TABLE 6

Hematopoietic and endothelial cell surface markers.

Flk-1	Vascular endothelial growth factor receptor expressed by early mesoderm, vascular progenitor, and endothelial cells.
CD34	Antigen expressed by hematopoietic stem, hematopoietic progenitor, and endothelial cells.
CD45	Antigen expressed by hematopoietic cells
CD31	Cell adhesion molecule expressed by platelets, endothelial cells, and murine ES cells.

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TABLE 7

Comparison of cell proliferation and differentiation between standard ES cell differentiation culture: liquid culture (LSC), and bioprocess of the invention: encapsulated stirred culture (ESC). Proliferation reported as total cells per EB. Flk-1 expressing mesoderm with hematopoietic potential was assayed on day 4 of differentiation culture. Hematopoietic progenitors coexpressing CD34 and CD45, and/or capable of hematopoietic colony formation (CFC) were assayed on day 7.

Analysis	LSC	ESC
Day 4 - Mesoderm		
Total Cells/EB	700±300	1200±450
Flk-1 ⁺ cells	44±12%	38±8%
Day 7 - Hematopoietic		
Total Cells/EB	3000±1500	3500±700
CD34 ⁺ CD45 ⁺ cells	1.9±1.2%	1.0±0.6%
CFCs per 10 ⁵ cells	14.0±6.0	5.0±2.0

TABLE 8

Comparison of hematopoietic cell development (on day 7 of differentiation culture) between normoxic (>160 mmHg O₂) and hypoxic (30-40 mmHg O₂) encapsulated stirred culture (ESC).

Analysis (Day 7)	Normoxic ESC	Hypoxic ESC
Total CD34 ⁺ cells	6.1%	8.0%
Total CD45 ⁺ cells	0.9%	2.6%
CD34 ⁺ CD45 ⁺ cells	0.6%	1.6%
CFCs per 10 ⁵ cells	5±2.0	7±2.6

TABLE 9

Cell Lineage-Specific Promoters

Promoter	Obtained Cell Lineage	Literature
β -cardiac myosin heavy chain promoter	cardiomyocytes	Klug et al. (1996) J. Clin. Invest. 98:216-24
MLC-2v	cardiomyocytes	Muller et al. (2000) FASEB J. 2000 Dec;14(15):2540-8
human atrial natriuretic factor (hANF) promoter	cardiomyocytes	Wu et al. (1989) J. Biol. Chem. 264:6472-79
SOX2 gene	neural precursors	Li et al. (1998) Curr. Biol. 8:971-4
Human insulin promoter (HIP)	Beta cells (insulin-secreting cells)	Soria et al. (2000) Diabetes 49:157
PDX1 promoter	Beta cells (insulin-secreting cells) pancreatic cells	Gannon M. et al. (2001) Dev Biol. 2001 Oct 1;238(1):185-201
Pax4 promoter	Beta cells (insulin-secreting cells)	Brink C. et al. (2001) Mech Dev. 2001 Jan;100(1):37-43, Smith SB. et al. (2000) J Biol Chem. 2000 Nov 24;275(47):36910-9.
VE-Cadherin promoter	Endothelial cells	Gory S. et al. (1999) Blood. 1999 Jan 1;93(1):184-92.
human VE-Cadherin-2 promoter	Endothelial cells	Ludwig D. et al. (2000) Mamm Genome. 2000 Nov;11(11):1030-3

TABLE 10

- 5 Examples of cell types derived from the in vitro differentiation of ES cells, as well as examples of additives thought to encourage the differentiation / survival of the specific cell types are indicated. In most cases the additive is added to serum containing media; examples of cells generated in a Chemically Defined Media (CDM) are also listed.

Cell Type Generated	Example Additives	Reference
Primitive ectoderm	CDM	Tropepe et al. 2001
Mesoderm precursors	BMP4, Activin A	Wiles et al. 1999
Endothelial cells	VEGF	Eichmann et al. 1997
Hematopoietic cells	IL-11+KL, IL-1 VEGF+KL VEGF+BMP4 IL-2+IL-3+Con A	Keller et al. 1993 Kennedy et al. 1997 Nakayama et al. 2000 Chen et al. 1992
Erythroid cells	Epo	Wiles et al. 1991
Myeloid cell	IL-3+IL-1+Epo, IL-3+IL-1+M-CSF / GM-CSF IL-3+M-CSF	Wiles et al. 1991 Lieschke et al. 1995
Lymphoid cells	VEGF+BMP4 RP.0.010+IL3+IL-6+IL-11	Nakayama et al. 2000 Gutierrez-Ramos et al. 1992
Skeletal muscle	TGF β 1 / TGF β c2 DMSO	Slager et al. 1993 Dinsmore et al. 1996
Cardiac muscle	TGF β 1 / TGF β 2 RA	Slager et al. 1993 Wobus et al. 1997
Neuroectoderm	CDM NGF bFGF	Tropepe et al. 2001 Yamada et al. 1994 Okabe et al. 1996
Neurons	RA	Bain et al. 1995
Adipocytes	RA+insulin+T3	Dani et al. 1997
Beta-cells	B27 Supplement & bFGF B27 Supplement & Nikotinamid	Lumelsky NO. et al.2001
Beta-cells	Nikotinamid	Soria B. et al. 2000
Beta-cells	EGF, HGF and Nikotinamid	Ramiva VKM. et al. 2000

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TABLE 11

Examples of cell types derived from the in vitro differentiation of human embryonic stem cells, as well as examples of additives thought to encourage the differentiation / survival of the specific cell types are indicated.

Cell Type Generated	Example Additives	Reference
Mesodermal cells	Activin-A and TGFbeta1	Schuldiner M, et al. 2000
Ectodermal, Mesodermal cells	Retinoic acid (RA), EGF, BMP-4, bFGF	Schuldiner M, et al. 2000
Ectodermal, Mesodermal Endodermal cells	NGF and HGF	Schuldiner M, et al. 2000
Neuronal cells	Retinoic acid (RA), nerve growth factor (betaNGF)	Schuldiner M, et al. 2001
Hepatocytes	sodium butyrate	Rambhatla L, et al. 2001
Beta-cells		Assady S, et al. 2001
Cardiomyocytes		Kehat I, et al. 2001
Endothelial cells		Levenberg S, et al. 2002

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

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WHAT IS CLAIMED IS:

1. A method of generating cells derived from spheroid-forming cells comprising culturing spheroid-forming cells under controlled cell aggregation conditions that enable spheroid formation in liquid suspension.
2. The method of claim 1 wherein the conditions enable spheroid formation in liquid suspension under high cell density.
3. The method of claim 1 or 2 wherein the cells are kept in liquid suspension by stirring.
4. The method of any one of claims 1-3 wherein controlled cell aggregation conditions means preventing spheroid aggregation.
5. The method of any one of claims 1- 4 wherein controlled cell aggregation conditions means permitting cell aggregation sufficient for spheroid formation and preventing aggregation between cells of different spheroids.
6. The method of claim 4 or 5 wherein the preventing aggregation is done by any one or more of the following means:
 - (a) preventing physical association between cell aggregation molecules;
 - (b) removing inorganic salts from the cell culture;
 - (c) blocking surface aggregation molecules with specific peptides;
 - (d) using spheroid-forming cells that are cell adhesion molecule-null spheroid-forming cells; and
 - (e) inhibiting cell adhesion molecule expression and/or activity.
 - (f) Methods that prevent cell aggregation by physically separating one or more spheroid-forming cells to enable spheroid formation in distinct compartments such as described in the single cell liquid suspension culture (scLSC) method in this patent without limitation to a single cell.
7. The method of any one of claims 1-6 wherein the controlled aggregation is affected by encapsulation of spheroid-forming cells.
8. The method of claim 7 wherein each capsule contains an initial number of spheroid-forming cells sufficient to enable spheroid formation.
9. The method of claim 8 wherein one spheroid is formed per capsule.

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10. The method of claim 8 wherein the initial number of cells is 1.
11. The method of claim 7 wherein the capsule initially comprises multiple cells of at least two different cell types.
12. The method of claim 11 wherein a spheroid is formed and the spheroid is a chimeric spheroid.
13. The method of claim 7 wherein the capsules have a capsule body comprising a matrix that permits passage of desired media constituents through the matrix to the spheroid-forming cells and spheroid, but do not permit aggregation with cells outside the capsule.
14. The method of 7 wherein the gel microdrop method is used to encapsulate the spheroid-forming cells.
15. The method of any one of claims 1-14 wherein the cells are cultured under conditions that promote cell expansion but not cell differentiation.
16. The method of any one of claims 1-14 wherein the cells are cultured under conditions that promote cell differentiation.
17. The method of claim 16 wherein the cells are cultured in an environment comprising one or more of the following: growth factors, cytokines, extra-cellular matrix molecules, drugs, low molecular weight molecules or compounds, retinoic acid, DMSO .
18. The method of claim 16 or 17 wherein differentiated spheroid-forming cells of interest are selected and harvested.
19. The method of claim 18 wherein the cells of interest are selected on the basis of cell surface markers.
20. The method of claim 18 wherein the differentiated cells of interest are selected from the group consisting of: cardiac myocytes, vascular progenitor cells, neurons, hepatocytes, glial cells, kidney cells, beta cells and derivatives thereof.
21. A method for the selection of specific cell types from spheroid-forming cells comprising the steps of:
 - i. introducing a reporter gene expressing vector into at least one spheroid-forming cell whereby a cell-type specific promoter is combined with an reporter gene, such that the reporter gene is expressed under the control of the cell-type specific promoter;

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- ii. culturing the spheroid-forming cell(s)
 - iii. differentiating spheroid-forming cell(s) in accordance with the method of claim 16
 - iv. isolating and harvesting the specific cell-type based on the reporter gene expression.
22. The method for the selection according to claim 21, wherein the reporter gene is an antibiotic resistance gene and the cell-type of interest is isolated by the addition of an appropriate antibiotic in step (iii) or/and (iv).
23. The method for the selection according to claim 22, wherein the antibiotic resistance gene is selected from but not limited to the group consisting of the Hygro mycin resistance gene (hph), the Zeocin resistance gene (Sh ble), the Puromycin resistance gene (pacA), and the Gentamycin of G418 resistance gene (aph).
24. The method for the selection according to claim 21, wherein the reporter gene is selected from but not limited to the group consisting of luciferase, green fluorescence protein, red fluorescence protein, and yellow fluorescence protein and the cells of interest are selected by fluorescent activated cell sorting (FACS).
25. The method for the selection according to claim 21, wherein the reporter gene is selected from but not limited to the group consisting of luciferase, green fluorescence protein, red fluorescence protein, and yellow fluorescence protein and the cells of interest are selected by fluorescent activated cell sorting (FACS).
26. The method for the selection according to claim 21, wherein the reporter gene is selected from but not limited to the group consisting of luciferase, green fluorescence protein, red fluorescence protein, yellow fluorescence protein, or a his-, myc-, or flag-tag ligated to a heterologous gene, or any heterologous gene which when expressed is inserted into the cell surface and the cells of interest are isolated from the cultured cells by affinity purification.
27. The method for the selection according to claim 21, wherein the cell type specific promoter is selected from but not limited to the group consisting of alpha-cardiac myosin heavy chain, MLC-2v, hANF, SOX2, HIP, PDX1,

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VE-Cadherin, human VE-Cadherin-2 and the selected cell type of interest is a cardiomyocyte, neural precursor, beta cell, endothelial cell.

28. The method of any one of claims 1-27 wherein the spheroid-forming cells are pluripotent cells.

5 29. The method of claim 28 wherein the pluripotent cells are selected from the group consisting of embryonic stem cells, embryonic germ layer cells, early primitive ectoderm-like cells, neural stem cells and adult pluripotent cells, adult mesenchymal stem cells, ductal setm cells, muscle derived stem cells, multiple adult progenitor cells.

10 30. The method of claim 29 wherein the pluripotent cells are embryonic stem cell and the spheroid is an embryoid body.

31. Embryonic stem cell derived cell culture obtained using the method of any one of claims 1-30.

15 32. The Spheroid-forming cell derived cell culture obtained using the method of anyone of claims 1-30.

33. A method to identify factors that effect cell proliferation, differentiation and/or spheroid formation, said method comprising culturing the cells as per the method of any one of claims 1-30 in the presence of the factor to be tested and then monitoring the effect of the factor on cell proliferation, differentiation and/or spheroid formation.

20 34. A method of generating cells derived from spheroid-forming cells in accordance with any one of claims 1-30 wherein the spheroid-forming cells are cultured under conditions that enable spheroid formation, said conditions comprising culturing said spheroid-forming cells in liquid suspension under non-aggregating conditions.

25 35. A culture bioreactor for industrial production of cells derived from spheroid-forming cells comprising:

- 30 c. culturing spheroid-forming cells in a spheroid-forming cell suitable media under conditions that promote spheroid formation; while
- d. Inhibiting spheroid aggregation.

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36. A method to prevent aggregation between cells comprising encapsulating a cell or group of cells and thus preventing aggregation of said cells with cells not within said capsule.

5 37. A bioreactor for generating cells from spheroid-forming cells comprising a means for controlling conditions suitable for spheroid formation, such conditions comprising a means for controlling cellular aggregation and means for maintaining said cells and generated cells in suspension.

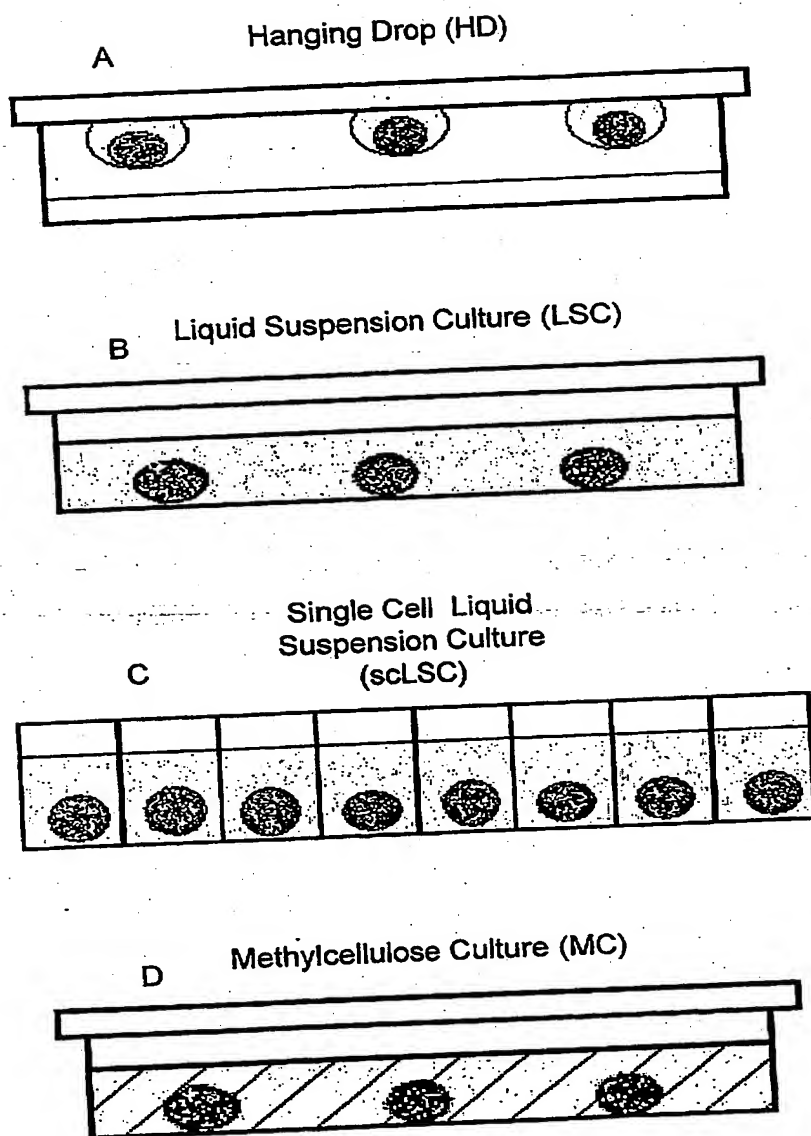
38. The bioreactor of claim 37 wherein said means for controlling cellular aggregation is means for preventing cellular aggregation.

10 39. The bioreactor of claim 37 or 38 which is seeded with at least one spheroid-forming cell.

40. The bioreactor of claim 37, 38 or 39 wherein at least one spheroid-forming cell is encapsulated.

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FIGURE 1

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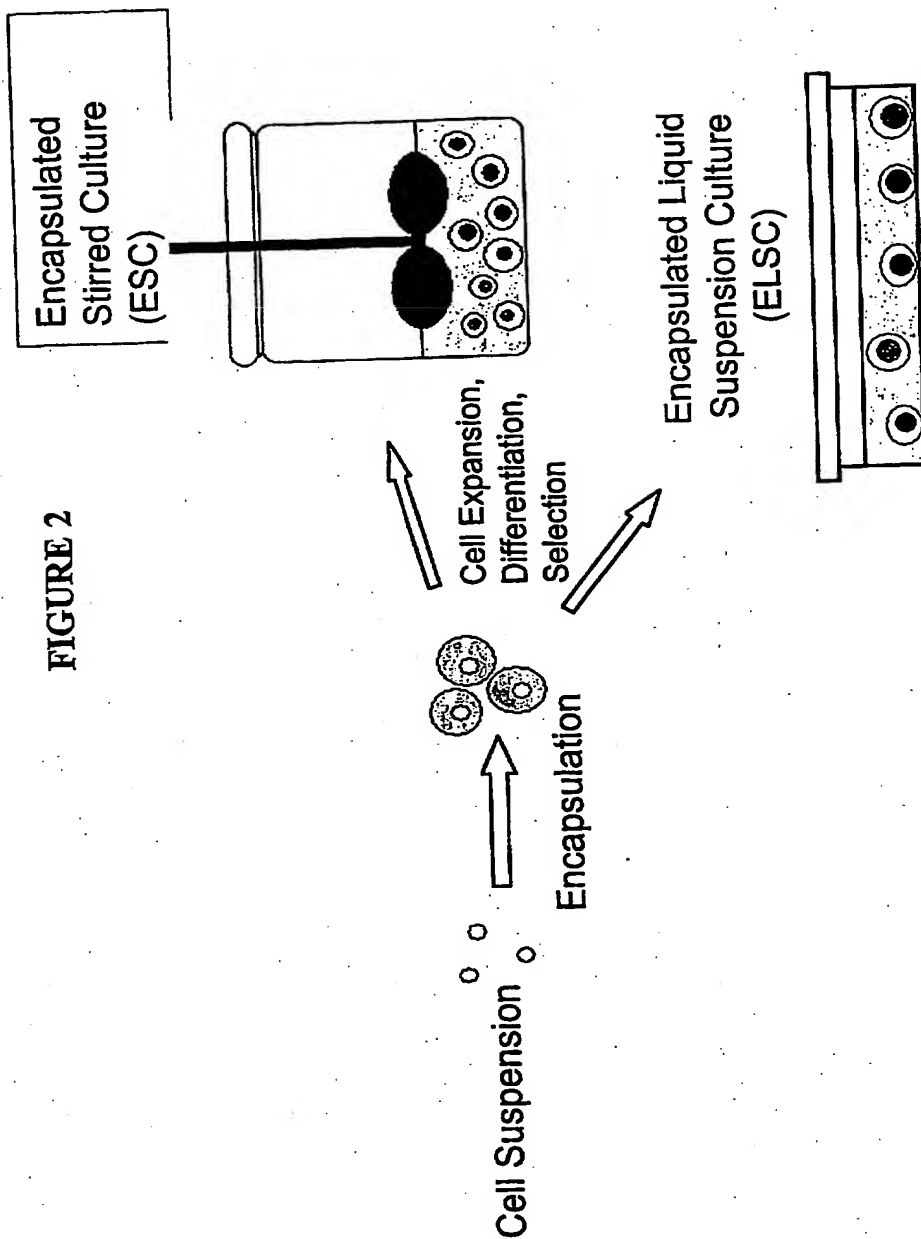
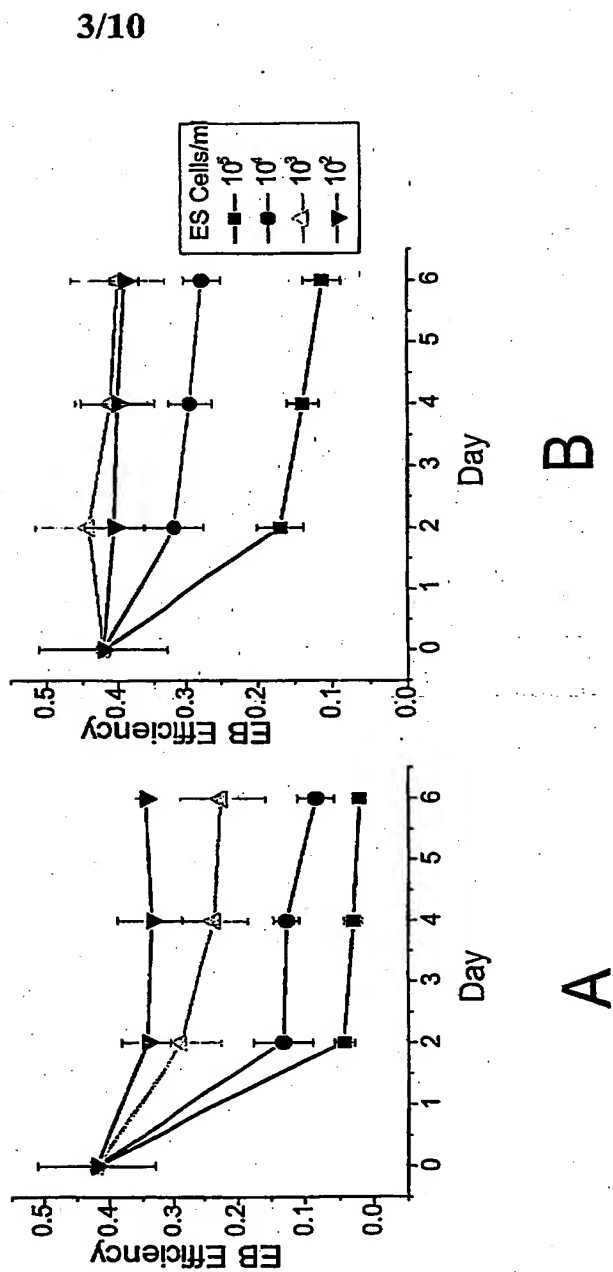
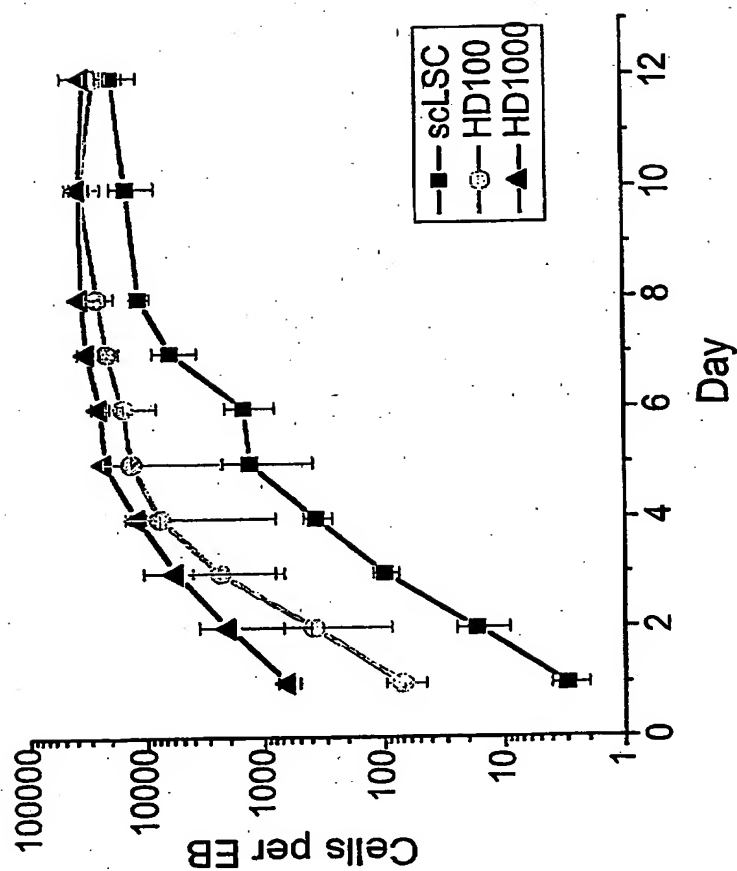


FIGURE 3



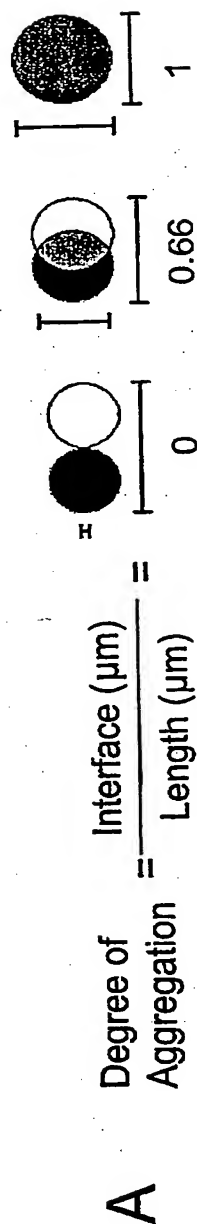
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FIGURE 4

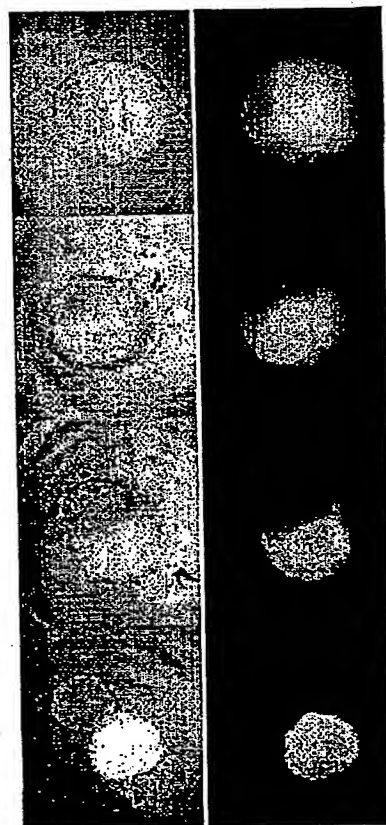


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FIGURE 5



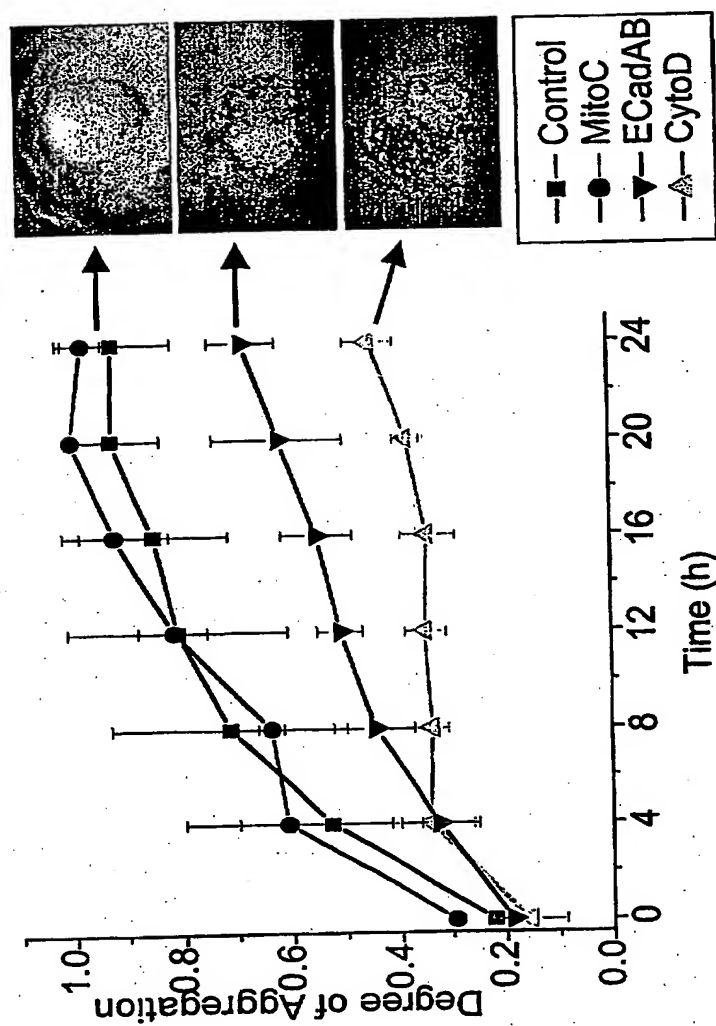
B Time 0h 12h 24h 48h



DOA 0.17 0.7 0.95 0.99

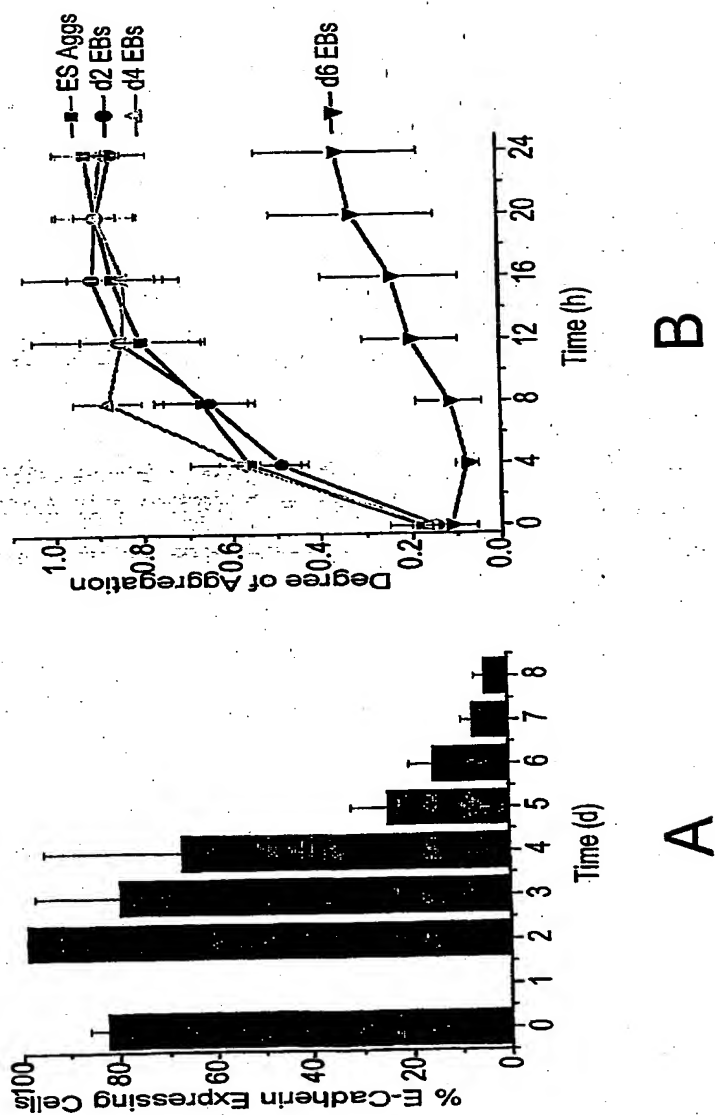
6/10

FIGURE 6



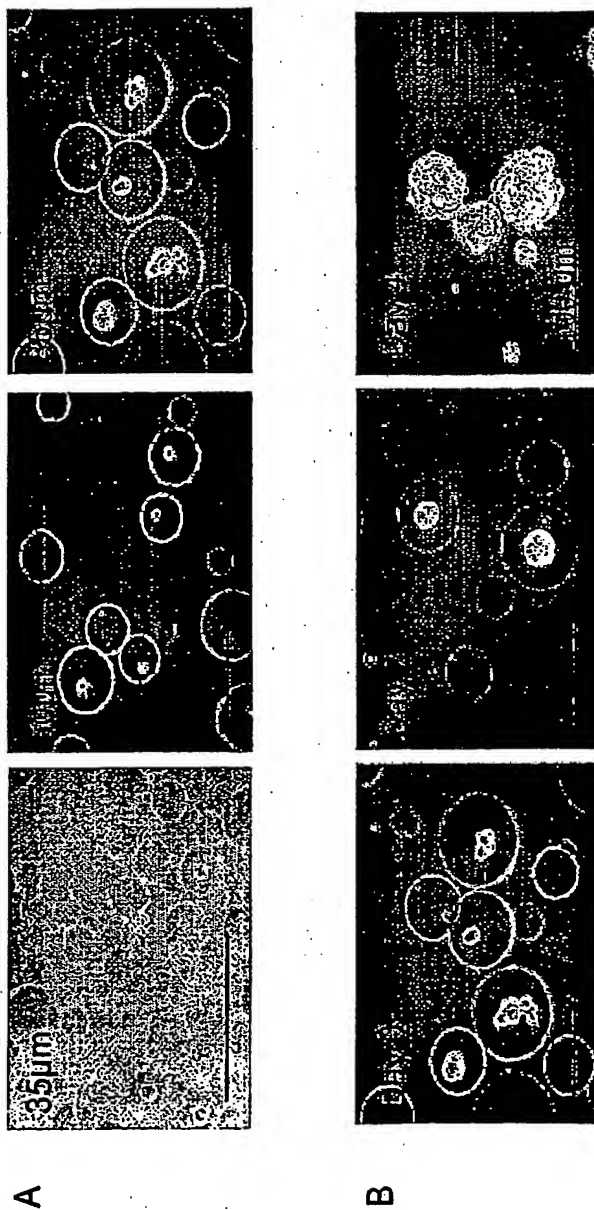
7/10

Figure 7



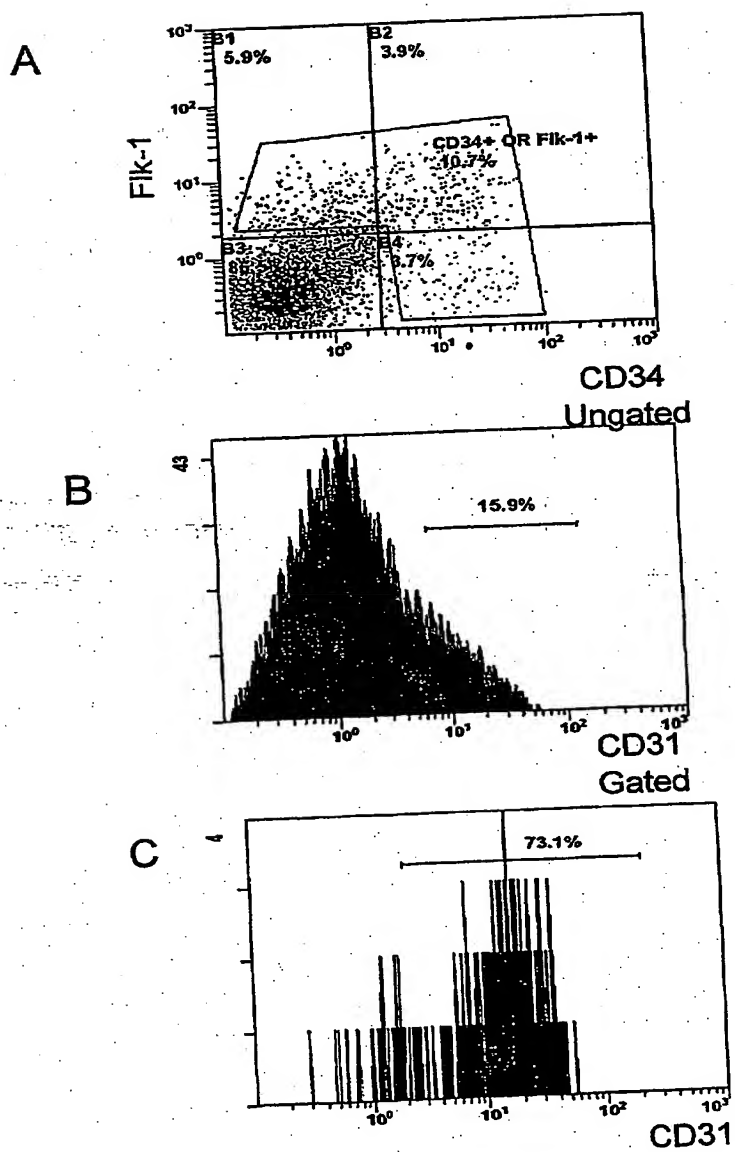
8/10

FIGURE 8



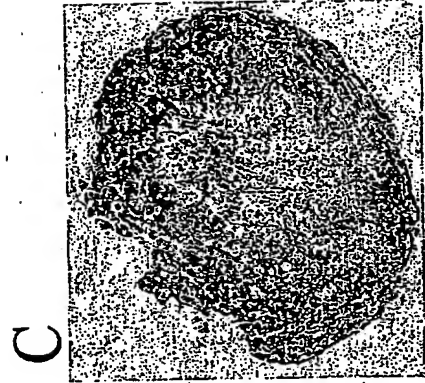
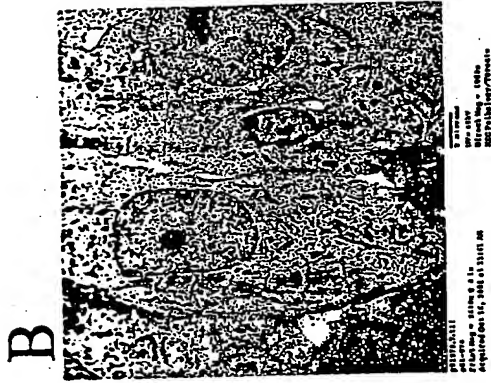
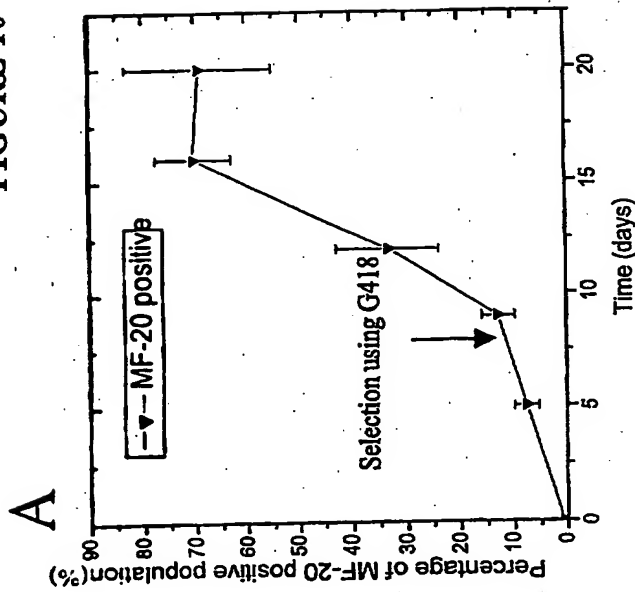
9/10

FIGURE 9



10/10

FIGURE 10



(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 January 2003 (16.01.2003)

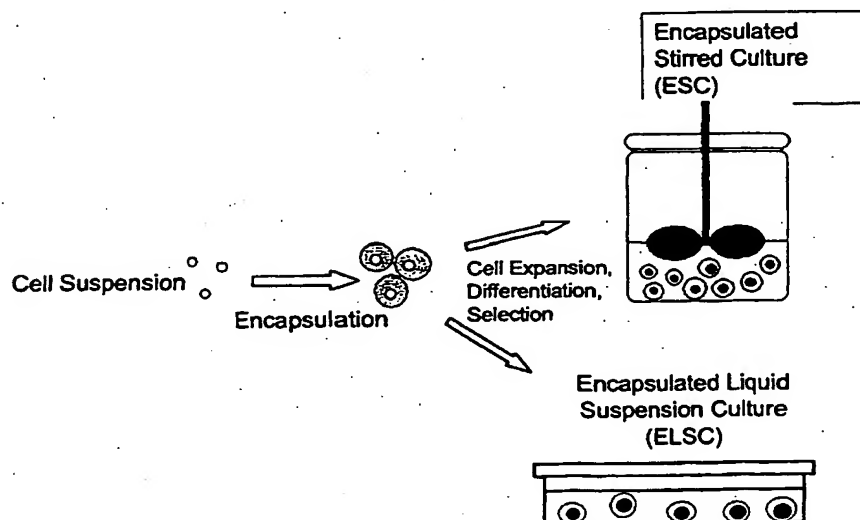
PCT

(10) International Publication Number
WO 03/004626 A3

- (51) International Patent Classification⁷: C12N 5/06, (74) Agent: BERESKIN & PARR; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).
C12M 3/02, A01K 67/027, C12N 15/62
- (21) International Application Number: PCT/CA02/01022 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (22) International Filing Date: 4 July 2002 (04.07.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
2,351,156 4 July 2001 (04.07.2001) CA
60/302,706 5 July 2001 (05.07.2001) US
10/189,642 3 July 2002 (03.07.2002) US
- (71) Applicant (*for all designated States except US*): CARDION AG [DE/DE]; IPR, Max-Planck-Str. 15a, 40699 Erkrath (DE).
- (72) Inventors: ZANDSTRA, Peter, W. [CA/CA]; 84 Alcina Avenue, Toronto, Ontario M6G 2E6 (CA). DANG, Stephen [CA/CA]; 58 Ambrose Road, North York, Ontario M2K 2W9 (CA).
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

[Continued on next page]

(54) Title: A BIOPROCESS FOR THE GENERATION OF CELLS DERIVED FROM SPHEROID-FORMING CELLS



(57) Abstract: The present inventors identified aggregation of embryonic stem cells and embryoid bodies (EBs) as the cause of the difficulty in generating large numbers of the embryonic stem cells (ES) cell-derived tissues. To counter this, the invention provides a novel bioprocess where aggregation of spheroid-forming cells, such as embryonic stem cells and spheroids, such as EBs is controlled, such as by encapsulation of within a matrix. As a result, EBs can be generated with high efficiency and cultured in high cell density, well-mixed systems. Well-mixed conditions facilitate measurement and control of the bulk media conditions and allow for the use of scalable bioreactor systems for clinical production of tissue. Therefore, the invention enables generation of ES cell-derived tissue on a clinical scale. The invention is also applicable to any spheroid-forming cells and other types of pluripotent cells.

WO 03/004626 A3



(88) Date of publication of the international search report:
15 May 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(15) Information about Correction:

Previous Correction:

see PCT Gazette No. 10/2003 of 6 March 2003, Section II

INTERNATIONAL SEARCH REPORT

Inter: Application No
PC1/LA 02/01022

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7¹ C12N5/06 C12M3/02 A01K67/027 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>DANG STEPHEN M ET AL: "Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems." BIOTECHNOLOGY AND BIOENGINEERING. UNITED STATES 20 MAY 2002, vol. 78, no. 4, 20 May 2002 (2002-05-20), pages 442-453, XP002219286 ISSN: 0006-3592 abstract page 444, left-hand column, paragraph 2 page 452, left-hand column, paragraph 3 -right-hand column, paragraph 3 --- -/-</p>	<p>1-6, 13-20, 28-32, 34</p>



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

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- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

5 November 2002

Date of mailing of the international search report

12.02.03

Name and mailing address of the ISA,

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Weiland, S

INTERNATIONAL SEARCH REPORT

Inten I Application No
PCI/CA 02/01022

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>MAGYAR JOSEF P ET AL: "Mass production of embryoid bodies in microbeads." ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 944, November 2001 (2001-11); pages 135-143, XP001118571 Conference on Bioartificial Organs, III: Tissue Sourcing, Immunoisolation, and Clinical Trials; Davos, Switzerland; October 07-11, 2000, Tissue sourcing, immunoisolation, and clinical trials. November, 2001 New York Academy of Sciences 2 East 63rd Street, New York, NY, 10021, USA ISBN: 1-57331-342-4 abstract page 136, paragraph 3 -page 137, paragraph 3 page 140, paragraph 1 -page 141, paragraph 1</p>	1-20, 28-32, 34,36
X	<p>COSBY N C ET AL: "MICROENCAPSULATION OF SINGLE MULTIPLE AND ZONA PELLUCIDA-FREE MOUSE PREIMPLANTATION EMBRYOS IN SODIUM ALGINATE AND THEIR DEVELOPMENT IN-VITRO" JOURNAL OF REPRODUCTION AND FERTILITY, vol. 90, no. 1, 1990, pages 19-24, XP001118759 ISSN: 0022-4251 abstract page 20, paragraph 5 page 21; example 1</p>	1-20, 28-32, 34,36
X	<p>PAPAS K K ET AL: "Cultivation of recombinant, insulin-secreting AtT-20 cells as free and entrapped spheroids." CYTOTECNOLOGY, vol. 13, no. 1, 1993, pages 1-12, XP001118058 ISSN: 0920-9069 abstract page 3, right-hand column, paragraph 2 -page 5, left-hand column, paragraph 1</p> <p style="text-align: center;">-/--</p>	1-17,32, 34,36

INTERNATIONAL SEARCH REPORT

Inten I Application No
PCT/CA 02/01022

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAKAI YASUYUKI ET AL: "A new bioartificial liver using porcine hepatocyte spheroids in high-cell-density suspension perfusion culture: In vitro performance in synthesized culture medium and in 100% human plasma." CELL TRANSPLANTATION, vol. 8, no. 5, 1999, pages 531-541, XP001118579 ISSN: 0963-6897 abstract page 532, left-hand column, paragraph 3 -page 533, right-hand column, paragraph 1 figure 2 ---	35,37-40
X	US 5 073 491 A (FAMILLETTI PHILIP C) 17 December 1991 (1991-12-17) abstract figure 1 column 2, line 44 - line 48 column 3, line 12 -column 5, line 48 ---	35,37-40
A	WARTENBERG M ET AL: "The embryoid body as a novel in vitro assay system for antiangiogenic agents." LABORATORY INVESTIGATION;A JOURNAL OF TECHNICAL METHODS AND PATHOLOGY. UNITED STATES OCT 1998, vol. 78, no. 10, October 1998 (1998-10), pages 1301-1314, XP001107032 ISSN: 0023-6837 cited in the application page 1308, left-hand column, paragraph 3 -right-hand column, paragraph 2 page 1311, left-hand column, paragraphs 1,2 figures 1-4 -----	1-20, 28-32, 34-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 02/01022

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-20, 31 and 32 (all in part)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20 and 35-40 (complete) and 28-32 and 34 (in part)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-20 and 35-40 (complete) and 28-32 and 34 (in part)

A method of generating spheroids from spheroid-forming cells wherein said spheroid-forming cells are encapsulated in a matrix like a polymer, e.g. agarose or alginate, and cultivated in a stirred liquid culture under high density, e.g. by means of the gel microdrop method, wherein each capsule initially contains one cell or multiple cells of at least two different cell types; said method wherein the cells are cultured in a medium comprising one or more of the following: growth factors, cytokines, extracellular matrix molecules, drugs, retinoic acid, DMSO; said methods wherein differentiated spheroid-forming cells - cardiac myocytes, vascular progenitor cells, neurons, hepatocytes, glial cells, kidney cells or beta cells - are selected, e.g. by means of cell surface markers, and harvested; said methods wherein the spheroid-forming cells are pluripotent cells - selected from the group consisting of embryonic stem cells, embryonic germ layer cells, early primitive ectoderm-like cells, neuronal stem cells, adult pluripotent cells, adult mesenchymal stem cells, ductal stem cells, muscle derived stem cells or multiple adult progenitor cells; said methods wherein the spheroid-forming cells are embryonic stem cells and the spheroid is an embryoid body; cultures of hematopoietic progenitor cells or endothelial cells derived from spheroid-forming cells, in particular embryonic stem cells; a bioreactor suitable for said methods.

2. Claims: 21-27 (complete) and 28-32 and 34 (in part)

A method for the selection of specific cell types from spheroid-forming cells whereby a reporter gene under the control of a cell-type specific promoter is introduced into at least one of the spheroid-forming cells, said cells are cultivated according to a method wherein they are encapsulated in a matrix like a polymer, e.g. agarose or alginate, and cultivated in a stirred liquid culture under high density, e.g. by means of the gel microdrop method, wherein each capsule initially contains one cell or multiple cells of at least two different cell types, and the specific cell-type is selected on the basis of the reporter gene expression; said method wherein said reporter gene is an antibiotic resistance gene, e.g. the resistance gene against Hygromycin, Zeocin, Puromycin or Gentamycin and the cells of interest are selected on the basis of the resistance to the corresponding antibiotic or wherein the reporter gene encodes e.g. luciferase, green, red or yellow fluorescence protein and the cells of interest are selected by fluorescent activated cell sorting or wherein the reporter gene is a heterologous gene expressed on the cell surface and the cells are selected on the basis of affinity purification; said method wherein the cell-type specific

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

promotor is e.g. the promotor of the gene encoding alpha-cardiac myosin heavy chain, MLC-2v, hANF, SOX2, HIP, PDX1, VE-Cadherin or human VE-Cadherin-2 and the selected cell type of interest is a cardiomyocyte, neural precursor, beta cell or an endothelial cell;

Said methods wherein the spheroid-forming cells are pluripotent cells - selected from the group consisting of embryonic stem cells, embryonic germ layer cells, early primitive ectoderm-like cells, neuronal stem cells, adult pluripotent cells, adult mesenchymal stem cells, ductal stem cells, muscle derived stem cells or multiple adult progenitor cells; said methods wherein the spheroid-forming cells are embryonic stem cells and the spheroid is an embryoid body.

3. Claim : 33

A method to identify factors that effect cell proliferation, differentiation and/or spheroid formation wherein the cells are cultured according to methods of the previously identified inventions in the presence of the factor to be tested and the effect of the factor on cell proliferation, differentiation and/or proliferation is measured.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-20, 31 and 32 (all in part)

Present claims 1, 2, 4 and 5 relate to a method defined by reference to a desirable characteristic or property, namely the formation of spheroids (1, 2) and the prevention of aggregation between cells of different spheroids (4, 5), resp.

The claims cover all methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the method by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the methods described in the examples, i.e. the generation of spheroids wherein the spheroid forming cells are encapsulated in a matrix like a polymer (e.g. agarose, alginate) and cultivated in a stirred liquid culture (Example 2b).

Claims 3, 6-20, 28-30 and 34 are dependent on one or more of claims 1, 2, 4 and 5. Therefore within the first group of inventions the search has been restricted to those parts of the claims 3, 6-20, 28-30 and 34 that refer to the searched subject-matter of claims 1, 2, 4, and 5, i.e. the methods concerning the generation of spheroids wherein the spheroid forming cells are encapsulated in a matrix like a polymer (e.g. agarose, alginate) and cultivated in a stirred liquid culture (Example 2b).

Moreover, present claims 31 and 32 relate to an extremely large number of possible products, namely cell cultures. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the products claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the products disclosed in the examples, i.e. hematopoietic progenitor cells and endothelial cells (Example 3 a and b).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 02/01022

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5073491	A	17-12-1991	NONE

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 January 2003 (16.01.2003)

PCT

(10) International Publication Number
WO 03/004626 A3

(51) International Patent Classification⁷: C12N 5/06,
C12M 3/02, A01K 67/027, C12N 15/62

Stephen [CA/CA]; 58 Ambrose Road, North York, Ontario M2K 2W9 (CA).

(21) International Application Number: PCT/CA02/01022

(74) Agent: BERESKIN & PARR; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).

(22) International Filing Date: 4 July 2002 (04.07.2002)

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2,351,156 4 July 2001 (04.07.2001) CA
60/302,706 5 July 2001 (05.07.2001) US
10/189,642 3 July 2002 (03.07.2002) US

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): CARDION AG [DE/DE]; IPR, Max-Planck-Str. 15a, 40699 Erkrath (DE).

(71) Applicants and

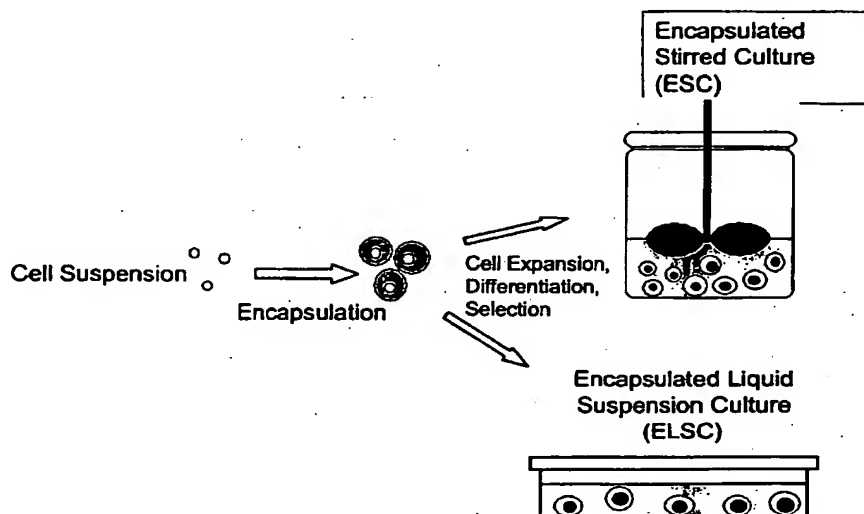
(72) Inventors: ZANDSTRA, Peter, W. [CA/CA]; 84 Alcina Avenue, Toronto, Ontario M6G 2E6 (CA). DANG,

Published:

— with international search report.

[Continued on next page]

(54) Title: A BIOPROCESS FOR THE GENERATION OF CELLS DERIVED FROM SPHEROID-FORMING CELLS



(57) Abstract: The present inventors identified aggregation of embryonic stem cells and embryoid bodies (EBs) as the cause of the difficulty in generating large numbers of the embryonic stem cells (ES) cell-derived tissues. To counter this, the invention provides a novel bioprocess where aggregation of spheroid-forming cells, such as embryonic stem cells and spheroids, such as EBs is controlled, such as by encapsulation of within a matrix. As a result, EBs can be generated with high efficiency and cultured in high cell density, well-mixed systems. Well-mixed conditions facilitate measurement and control of the bulk media conditions and allow for the use of scalable bioreactor systems for clinical production of tissue. Therefore, the invention enables generation of ES cell-derived tissue on a clinical scale. The invention is also applicable to any spheroid-forming cells and other types of pluripotent cells.

WO 03/004626 A3



— *with amended claims*

(88) Date of publication of the international search report:
15 May 2003

Date of publication of the amended claims: 17 July 2003

(15) Information about Correction:

Previous Correction:

see PCT Gazette No. 10/2003 of 6 March 2003, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

AMENDED CLAIMS

**[Received by the International Bureau on 11 April 2003 (11.04.03):
original claim 1 amended (1 page)]**

1. A method of generating cells derived from pluripotent cells comprising culturing pluripotent cells under conditions that control cell aggregation in a well mixed system, that enable spheroid formation in liquid suspension, and promote cell growth and/or cell differentiation.
2. The method of claim 1 wherein the conditions enable spheroid formation in liquid suspension under high cell density.
3. The method of claim 1 or 2 wherein the cells are kept in liquid suspension by stirring.
4. The method of any one of claims 1-3 wherein controlled cell aggregation conditions means preventing spheroid aggregation.
5. The method of any one of claims 1- 4 wherein controlled cell aggregation conditions means permitting cell aggregation sufficient for spheroid formation and preventing aggregation between cells of different spheroids.
6. The method of claim 4 or 5 wherein the preventing aggregation is done by any one or more of the following means:
 - (a) preventing physical association between cell aggregation molecules;
 - (b) removing inorganic salts from the cell culture;
 - (c) blocking surface aggregation molecules with specific peptides;
 - (d) using spheroid-forming cells that are cell adhesion molecule-null spheroid-forming cells; and
 - (e) inhibiting cell adhesion molecule expression and/or activity.
- (f) Methods that prevent cell aggregation by physically separating one or more spheroid-forming cells to enable spheroid formation in distinct compartments such as described in the single cell liquid suspension culture (scLSC) method in this patent without limitation to a single cell.
7. The method of any one of claims 1-6 wherein the controlled aggregation is affected by encapsulation of spheroid-forming cells .
8. The method of claim 7 wherein each capsule contains an initial number of spheroid-forming cells sufficient to enable spheroid formation.
9. The method of claim 8 wherein one spheroid is formed per capsule.

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